Aryadeep Roychoudhury *Editor*

Rice Research for Quality Improvement: Genomics and Genetic Engineering

Volume 2: Nutrient Biofortification and Herbicide and Biotic Stress Resistance in Rice

Springer

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Foreword

The significant yield loss of rice in response to abiotic stress and development of resilient smart rice crop has been described and documented in Volume I of the series *Rice Research for Quality Improvement: Genomics and Genetic Engineering*. Volume II of the series highlights broadly the biotic stress, herbicide resistance, and bioengineered biofortification in rice.

Biotic Stress Resistance

Rice is constantly exposed to interaction with various organisms and subject to important diseases. An annual loss between 120 and 200 million tons of grain due to insects, diseases, and weeds in rice fields in tropical Asia is well documented. Biotic factors that damage paddy crop are virus, bacteria, fungi, nematodes, and insect pests. Many morphological, anatomical, physiological, and biochemical factors have been reported to be associated with resistance, each controlled by different sets of genes. Molecular breeding is used for development of resistant varieties by changes in the genetic background of promising lines through the introduction of a new resistance gene. Plant breeders' and geneticists' attempts to produce new varieties that better tolerate pathogen attack have not resulted in any satisfactory cultivar, particularly sheath blight-resistant rice. Consequently, there exists a high demand for novel efficient genomics and biotechnological methods for controlling plant diseases, as well as for producing plants of interest with increased resistance to biotic stress. The ability to maintain or increase rice production in a cost-effective manner will rely on developing varieties that can be productive in response to a variety of abiotic or biotic stresses. Overexpression of genes or RNA interference (RNAi) to knockout the expressions of genes proves helpful to understand the biological functions of genes and encoded proteins. Additional research in this field has been focused on a number of areas including siRNA, microRNAs, hairpin RNA, and promoter methylation.

Herbicide-Tolerant Rice

Weedy rice (*Oryza sativa*), a conspecific weed of cultivated rice, is also a global threat to rice production. Classified as the same species as cultivated rice, it is highly competitive, is difficult to control without damaging cultivated rice, and can often cause total crop failure. The competition of cultivated rice with weedy rice can lead to yield losses from less than 5% to 60%. Hence, there is a growing need for generating herbicide-resistant rice particularly with the advantage of using direct seeded rice (DSR) due to labor shortage and use of efficient mechanized rice cultivation.

Biofortification

The polished rice devoid of micronutrients may satisfy hunger, but there is a deeper problem of "hidden hunger" which is only fulfilled by nutritionally enriched food. Having a balanced diet is a far-fetched dream for the underprivileged people of the world. A carbohydrate-rich diet including rice, wheat, or maize (the major staple food) is consumed worldwide and mainly contributes to solving the problem of hunger; however, malnutrition still persists in the world. "Hidden hunger" is caused when the body is deprived of essential micronutrients. Nutrient deficiency or malnutrition has affected at least 2 billion people (or 1 out of 3), mostly in Africa, South Asia, and Latin America. Micronutrient deficiency is a silent epidemic condition—it slowly weakens the immune system, stunts physical and intellectual growth, and even causes death. Among micronutrient deficiencies, iron deficiency or iron deficiency anemia (IDA), zinc deficiency, and vitamin A deficiency (VAD) are widespread and cause serious consequences. More than 24,000 people globally die daily owing to "hidden hunger" and malnutrition. To combat these deficiencies, fortification of food with different biological and chemical supplements and the alterations of the food processing system are essential. Biofortified (including bioengineered) staple food crop is a sustainable alternative that can be highly beneficial for people who have limited access to varied dietary resources. Genetically modified (GM) rice, particularly pro-vitamin A rice engineered with three genes driven by endosperm-specific promoters, expressing in endosperm known as "Golden Rice," and similarly engineered high iron rice with *ferritin* gene, may meet to fight against "hidden hunger."

Male Sterility

Male sterility has been tested as a specific mechanism to contain gene outflow in various crops; the transgene of choice is transformed into the male sterile (female) inbred to form a hybrid and is much discussed as a containment method. A great deal of male sterility is cytoplasmic and inherited on the chondriome (mitochondrial genome). This will be difficult to perform as chondriome engineering is yet

unknown. The ability of cytoplasmic male sterility to preclude transgene flow through pollen (using non-transgenic pollinators) seems helpful to decrease the risk of viable pollen flow, as tested under field conditions.

Rice Aroma

Fragrance in rice grain is the most attractive trait that fetches a premium price in the market. Continuously increasing demand for fragrant rice in global market has gained the special attention of rice breeders and forced them to consider rice grain aroma among major objectives for commercially improved rice varieties. Since aromatic varieties are very rare, these may be considered among the most precious treasures of India and can also be considered as national asset and pride. The fragrance of rice plays an important role in affecting the market value and consumers' preference. Recent advancements in plant science and availability of high-density linkage maps and fully sequenced rice genome have provided better opportunities for plant scientists to look inside the secrets of aroma in rice. Hence, maintenance of aroma in rice is a significant feature and is a vital objective for breeding rice cultivars for their commercial importance for the global market.

Flowering, Nitrogen Assimilation, and Particularly Grain Yield

Grain yield is related to the development of reproductive organs/tissues. A large number of genes are involved in the panicle development, fertilization, and seed development, including increased seed size. Analysis of gene clusters and networking would unravel the organ development and its linkage with encoded proteins and regulatory elements. Unfilled grains of most rice cultivars remained a challenging issue to be solved. ADP-glucose pyrophosphorylase (ADPPP) is referred to as limiting resource for starch biosynthesis and transport to grain. However, much more study is required to analyze this phenomenon and to find the coordination of nitrogen assimilation in the growing rice plants and particularly in the reproductive organs, and its effective function to drive the ADPPP. The authors have summarized some of the key issues in this volume.

Policy Making and Biosafety on Transgenic Rice

Overall, Volume 2 of this book has 34 chapters, encompassing all the above aspects. The first ten chapters focus mainly on rice resistance to pathogen infection and herbicides. The next few chapters highlight development of male sterile rice lines, understanding the genetic basis of flowering response in rice, improving water and nitrogen use efficiency, and dissecting the genetic basis of aroma production in rice. A major section of this volume is devoted to the biofortification approaches to enhance several nutrients in rice, and a large number of chapters have been

incorporated in this regard. There are a number of well-documented reviews worldwide which reveal that GM rice with inserted genes does not have any additional risk compared to traditionally developed improved rice. GM rice or GM crops so far commercialized do not have any special safety concerns. The public in some countries particularly in Europe is still skeptical regarding the consumption of GM food, and wide acceptance of such crops is only possible through adequate field trials and removal of public misconceptions. A chapter covering this aspect has therefore been included at the end. The only well-accepted hybrid Bt rice was developed at IRRI by the group of Dr. S.K. Datta and field-evaluated in China (Nature Biotechnology, 2000: 18:1101–1104) with the collaboration of Dr. Qifa Zhang and eventually marketed and consumed in China. Such examples of successful story in GM rice with improved traits are necessary for the consumers and economic benefits of the farmers and overall growth of agri-industry.

All the chapters on the abovementioned subjects are well written by the eminent authors and reviewed by the experts and finally edited by Dr. Aryadeep Roychoudhury, a well-known rice scientist. This book would be a valuable asset for the library, students, teachers, and scholars of different fields and particularly in rice research.

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Swapan K. Datta, Ph.D., is currently holding the DBT Distinguished Biotechnology Research Professor position at the Department of Botany, University of Calcutta, where he also served as Rashbehari Ghosh Chair Professor. Dr. Datta received B.Sc. (Hons.) from Presidency College (1972), M.Sc. (1974) and Ph.D. (1980) from the University of Calcutta. Dr. Datta received DAAD fellowship while working at Visva-Bharati University (1985–1986) and worked in Germany with Prof. G. Wenzel on resistance genetics of wheat and barley. Dr. Datta was awarded the Friedrich Miescher Institute (FMI) Fellowship attached to CIBA-GEIGY at Basel, Switzerland (1987). He took the senior scientist position at Swiss Federal Institute of Technology (ETH), Zurich, Switzerland (1987–1993) and worked on Gene technology for crop improvement with Prof. Ingo Potrykus, being associated with International collaborators. Dr. Datta was awarded Rockefeller Foundation supported Senior Visiting Faculty at UC-Davis, USA (1989) before joining at International Rice Research Institute (IRRI), Manila, Philippines where he contributed significantly to rice improvement with Dr. Gurdev Khush and with many International collaborators (1993–2005). Dr. Datta is a recipient of many national and international fellowships/awards including TATA Innovation Fellowship (2007–2009), CGIAR-best science research paper, and Paul Johnnes Brouhl Memorial Medal (2009). Attached with **Professional affiliations:** Elected Fellow of the Indian National Science Academy in 2014 (FNA), Elected Fellow of the Indian Academy of Science in 2017 (**FASc**), Elected Fellow of the National Academy of Agricultural Science in 2005 (**FNAAS**), Elected Fellow of National Academy of

Sciences in 2006 (**FNASc**), Elected Fellow of the World Academy of Science in 2014 (**TWAS**), Dr. Datta contributed significantly to *Rice research and traits improvement* over 40 years of research in Agriculture and Plant Biology, particularly on genetic transformation system in rice, jute, and chickpea, which has been established with landmark crop improvement. He has published over 150 research papers appearing in reputed journals including *Nature*, *Science*, *Nature Biotechnology*, and *Nature Genetics* with the contributions from 38 Ph.D. students and post-doctoral fellows and a large number of international collaborations.

Preface

Volume I of this book was mostly focused on the diverse environmental stresses encountered by rice plants and the means to tackle them using genomics approaches and genetic engineering to develop abiotic stress-tolerant rice. Environmental or climatic fluctuations, which are beyond control by humans undoubtedly appear to be the most severe challenge to any crop species like rice and hence deserve the most attention in terms of rice productivity. However, rice plant is also susceptible to infections by a host of pathogens like bacteria, fungus, insects, nematodes, and herbivores, as well as pathogen-induced wounding. Turning back the pages of history will show that the Bengal famine of 1943 was actually due to a disease called brown spot of rice caused by the fungus *Helminthosporium oryzae*, which ravaged fields of rice, leaving millions of people impoverished and to suffer or die from acute hunger. Blast disease, caused by the fungus *Magnaporthe oryzae*, and bacterial blight, provoked by the bacterium *Xanthomonas oryzae pv. oryzae*, represent two of the most destructive diseases of rice. The dissection of several signaling molecules inducible by the pathogens as a result of host–pathogen interaction, along with whole genome transcriptome analyses, proteomic studies, posttranslational modifications, and microRNA analyses, provides platforms for finding out novel genes for targeting in infected rice plants through either overexpression or microRNA technology-dependent downregulation. Unlike abiotic stress which is essentially a multigenic trait, governed by simultaneous regulation of a gene cascade, biotic stress can more often be controlled by genetic engineering of a single gene, e.g., *Xa21* overexpression generating rice lines resistant to bacterial blight. Apart from pathogen resistance, rice growers all over the world would largely benefit from development of herbicide-resistant rice cultivars which would eliminate weedy rice species and provide the possibility of selective control of "hard-to-kill" weeds evolving resistance to herbicides used in rice fields.

Rice, as a food crop, is the major source of carbohydrates. The aleurone layer of dehusked rice grain, which is rich in micronutrients, is lost during polishing so that rice grains we consume lack vitamins, micronutrient cations, and antioxidants. Therefore, consumption of rice from the dietary point of view can mitigate hunger, but does not resolve the problem of 'hidden hunger'. Hence, it is necessary to biofortify the nutrient content in polished rice grains through gene pyramiding by

overexpression of genes encoding multiple enzymes in a biosynthetic pathway for a particular metabolite. On the other hand, RNA interference-mediated approaches to lower down the level of anti-nutrients have also been adopted in certain cases to improve the nutrition level in rice grains. Such work definitely holds immense importance from the humanitarian point of view in achieving food security, since poor people of developing nations cannot afford to have supplemented diversified foods and hence solely rely on nutrient-enriched staple food crop to overcome malnutrition.

Improvement of rice quality in terms of other physiological traits like flowering, water and nitrogen use efficiency, and male sterility also appears to be extremely important. Flowering is directly related to seed production and grain yield. Male sterility is a useful agronomic trait for producing F_1 hybrids in self-pollinating crops like rice, which eliminates the tedious emasculation technique. These beneficial traits also involve elaborate genomics studies and breeding techniques or genetic engineering tools to come up with superior, value-added rice. Another desirable trait found in aromatic rice is the desirable fragrance, which is attributed to a host of volatile compounds, of which 2-acetyl-1-pyrroline is the predominant one. Efforts are being made to downregulate betaine aldehyde dehydrogenase 2 (BADH2) through RNA interference, so as to enhance aroma level in rice.

Volume 2 of this book has 34 chapters which revolve around all the above aspects. The first ten chapters focus mainly on rice resistance to pathogen infection and herbicides. The next few chapters highlight development of male sterile rice lines, understanding the genetic basis of flowering response in rice, improving water and nitrogen use efficiency, and dissecting the genetic basis of aroma production in rice. A major section of this volume is devoted to the biofortification approaches to enhance several nutrients in rice, and a large number of chapters have been incorporated in this regard. Developing genetically modified (GM) rice also brings in the biosafety issues together with the commercialization and public acceptance of the transgenic crops. The public is still skeptical regarding the consumption of GM rice, and wide acceptance of such crops is only possible through adequate field trials and removal of public misconceptions. A chapter covering this aspect has therefore been included at the end.

I strongly feel that this volume, like the earlier one, will be in great demand for the students, research scholars, teachers, and scientists working on diverse areas of rice improvement, at research institutes, universities, and colleges all over the world. It will also be helpful to the multinational companies involved in agricultural development or seed production.

I am immensely grateful to all the contributors and academicians involved in rice research who have been highly instrumental in making this volume a success. I pay respect to all my teachers who taught me the basics of plant science and created my interest in the field of rice research. I acknowledge the support and encouragement of the Principal, St. Xavier's College (Autonomous), Kolkata, where I am currently

working. I thank all my family members for their continued support and patience in my academic pursuits. I appreciate the cooperation and support of Springer Nature, which largely helped me to handle this Herculean task of editorial work single-handedly. Finally, I surrender myself totally to Almighty God who has always instilled in me the strength and power to trudge this struggling path of life against all adversities.

Kolkata, West Bengal, India Aryadeep Roychoudhury

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Understanding the Mechanism of Host-Pathogen Interaction in Rice Through Genomics Approaches

Yogita N. Sarki, Riwandahun Marwein, Sanjay Singh, Hariprasanna Dekaboruah, Dhanawantari L. Singha, and Channakeshavaiah Chikkaputtaiah

Abstract

Rice is one of the major food crops of the world, and the productivity of rice is greatly affected by biotic stress factors or pathogens such as fungi, bacteria, viruses and insect pests. Understanding the mechanism of host-pathogen interaction in rice is essential for designing various strategies for crop improvement and to sustain the crop productivity under rapidly changing global climate. The development of the novel state-of-the art tools and techniques in the post-genomics era has greatly enhanced our understanding of molecular and genetic mechanism of host-pathogen interaction in rice. Genomics datasets provide quick and better insight of infection biology, virulence, survival, colonization, and evolution. In this book chapter, we discuss the standard genomics approaches used for understanding the host-pathogen interaction in rice covering various genome-wide association studies including GBS, QTLs, association mapping of R genes, GW meta-analysis and SNP chip techniques. We also describe the role of epigenomics in gene expression reprogramming during biotic stress. A brief idea has been adumbrated regarding the latest NGS tools such as in vivo iPOOL-sequencing for identification and characterization of virulence factors and host-pathogen sequences. Also, a latest next-generation genomics approach has evidenced the emerging role of post-transcriptional regulator in host-pathogen

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biology such as miRNA and siRNA. In a nutshell, this chapter emphasizes on the most recent advancements in the field of genomics to have deeper understanding on host-pathogen interaction mechanism. This would be implied in designing futuristic strategies for sustainable crop productivity of rice.

Keywords

Functional genomics · Biotic stress · Rice · GWAS · NGS · Molecular markers · Defence mechanism · *Magnaporthe oryzae* · R genes · QTL mapping

1 Introduction

1.1 Rice

Rice is a significant cereal crop, and its importance is determined by the fact that it is the staple food crop for fifty percent of the world population living in Asia. It is estimated that by the year 2025, the world population will increase to eight billion. Hence to provide for the alarming growing population, global food and calorie demands, the total food production has to be increased by up to 50% (Khush 2005). Approximately rice is cultivated over 92% of total acreage and fulfils more than 23% calorie needs of people worldwide (Sharma et al. 2012). The rice cultivation faces a rigorous yield loss every year due to the biotic and abiotic stresses, so to overcome or at least decrease the effect of the environmental stress has been a crucial measure in order to meet the demands of the growing world population (Das and Rao 2015). Rice crop genome was first sequenced in 2005 during the International Rice Genome Sequencing Project. Thus it is uniquely poised as a model crop species, with ample genetic variation and the availability of genomics and phenotypic resources publicly.

1.2 Host-Pathogen Interaction: Molecular and Genetic Mechanism

To understand the strategies of host resistance and pathogen infection, numerous insights have been revealed by the genetic and molecular scrutinies of plant disease. Similar to the animals, plants also possess a defence mechanism that apprehends and responds to a broad spectrum of pathogenic microorganisms (Dodds and Rathjen 2010). There are multiple barriers in the defence system of plants. The outer barrier includes waxy cuticles, and the internal barriers refer to the resistance and defence response genes. The defence system in plants can be classified into two classes, basal defence and specific defence systems. (Chisholm et al. 2006). The basal defence line also known as innate immunity scrutinizes the pathogen's entry in the plants and imparts immunity at the onset of infection. It acts as a first line of defence which does

not distinguish between pathogens. This defence system is much effective against necrotrophic pathogens.

On the other hand, the specific defence system that is mediated by resistance (R) genes is effective against hemibiotrophs and biotrophs. The R genes limit the growth of pathogen and development of disease at the site of infection through the deployment of hypersensitive response (HR) developed by apoptosis. After the infiltration of first line of defence, this mechanism comes into action to dominate the pathogenic attack (Zipfel and Felix 2005). This interaction is a multifarious mechanism, mediated by the molecules derived from the pathogen and the plant that mainly includes proteins, sugars and lipopolysaccharides (Boyd et al. 2013). The apoplastic region is the first site of encounter between the plants and microbes. This encounter is mediated when the microbial elicitors are recognized by the receptor proteins present on the plants (Dodds and Rathjen 2010). Plants have a two-layered innate immune system: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI).

After the successful entry inside the host plant cell, the pathogens release certain molecules known as pathogen-associated molecular patterns (PAMPs) or microbial-associated molecular pattern (MAMPs). Few examples of PAMPs are bacterial flagellin proteins, peptidoglycan and ergosterol lipopolysaccharide. These microbial molecules are responsible for the pathogenicity of the microbes enabling them to successfully colonize the host cell. Parallelly, plant recognizes the PAMPs via pattern recognition receptors (PRRs) that are extracellular, transmembrane receptors. Further the defence response is elicited, and a relatively weak immune response is triggered. Hence colonizing of invading microorganisms is inhibited. However when the pathogens damage the host cells, certain native molecules of the host are released that are detected by PRRs referred to as damage-associated molecular patterns (DAMP) (Nicaise et al. 2009). Generally, when the PRRs recognize MAMPs/PAMPs or DAMPs, PTI is activated. This results in series of events like reactive oxygen species (ROS) is produced, mitogen-activated protein (MAP) kinase pathway is initiated, and several transcription factors are activated that totally restrict the advancement of pathogens (Nurnberger et al. 2018).

PRRs are plasma membrane-localized receptors that belong to two classes: receptor-like kinase (RLK) and receptor-like protein (RLP) (Zipfel 2008). RLK includes three domains: a single pass transmembrane domain, a cytoplasmic kinase domain and an ecto-domain (Shiu and Bleecker 2001). RLPs are similar in structure and function to Toll-like receptors (TLR) that are present in animals. Unlike RLK, RLP lacks kinases region (Ronald and Beutler 2010). The leucine-rich repeats (LRRs), in the extracellular ligand-sensing domain of PRR (Macho and Zipfel 2014), bind to peptides like bacterial elongation factor-Tu or flagellin (Chinchilla 2006). Apart from RLK and RLP that are involved in the recognition of protein or peptides, some PRRs recognize molecules containing sugars or carbohydrates via lectin motifs, lysine motifs (LysMs) or epidermal growth factor (EGF)-like domains. These domains recognize bacterial peptidoglycans, fungal chitin, plant cell wall-derived oligogalacturonides, etc. (Choi et al. 2014).

Plants being sessile are more affected by environmental factors both abiotic and biotic. Hence RLP and RLK are present in higher numbers than animals. Li et al. (2016) have reported a total of 90 RLPs and 640 RLKs in rice crop. Despite of having all these lines of defence in plants, various pathogen-released molecules are successful in manipulating the innate immunity of the plants and even remain undetected by the host immune system. ETI (effector-triggered immunity) is the second layer of innate immunity in plants. It is a fast and robust response in contrast to PTI. The basis for ETI is highly polymorphic resistance (R) genes. The domains present in the R genes are leucine-rich repeat (LRR) and nucleotide binding site (NBS). It is usually associated with a hypersensitive reaction (HR) (Jones and Dangl 2006). The activation of R genes occurs when they encounter the highly diverse avirulence effectors (Avr) derived from the pathogens. The innate immune response along with the mechanism of immunity provided by R genes is interceded by a complex network of signalling pathways that activates the genes involved in defence response. These genes include reactive oxygen species (ROS), genes related to pathogenesis, glucanases, secondary metabolites, chitinases, stomatal opening and closing, callose and lignin formation. These defence genes expressed against the bacterial and fungal pathogens anticipate in regulating at both protein and mRNA level. But in case of a viral invasion, they generally act at the RNA level only.

1.3 Host-Pathogen Interaction in Rice: Understanding the PAMP-ETI against Fungal Pathogens of Rice Using Magnaporthe Oryzae as a Model

Rice blast disease has been impacting many rice-producing countries in Asian and African continent. *Magnaporthe oryzae* (rice blast fungus) being the major culprit in the reduction in rice production is listed among the ten most important fungus that causes disease in plant (Dean et al. 2012). To gain a broad overview of the ongoing research problems in plant pathology, the study of rice blast fungus provides a coherent system to demonstrate most of the important concepts governing plant-fungal interactions (Wilson and Talbot 2009). The disease alone can destroy the annual yield of rice production that could be fed by 60 million people (Skamnioti and Gurr 2009). Recently, adequate information has been acquired with respect to the recognition and signalling of PTI and ETI in the *M. oryzae*-rice interaction. Maciel et al. (2014) reported that *M. oryzae* has broaden its host range by infecting wheat crop as well. This has been a threat in countries like Paraguay, Southern Brazil and Bolivia. Therefore it clearly exhibits the potential of rice blast fungus to infect the other crops of the grass family, causing diseases in them. The fungus is facing selection pressure resulting in the host jumps due to extensive cultivating of the cereals. Thus the development of basic understanding of the disease is mandatory in order to imply new strategy for long-lasting crop protection.

1.3.1 Disease Cycle of Magnaporthe Oryzae

The arrangement of conidia is in a sympodial manner on the aerial conidiophores and forms the inoculum for the secondary infection. Conidia are attached to the plant's surface where mucilage is stored in a section at the spore tip resulting in the germination and production of hyphae, branching filament that later makes up the mycelium of the fungus. Hyphae remains enclosed in an extracellular matrix that coheres to the surface. The formation of the dome shaped, melanized appressorium occurs. Further, the plant cell wall and the cuticle are penetrated by the turgor pressure employed by the penetration peg. This leads to rise of infection hyphae that have a protuberant appearance and dissociate inside the cell. Development of infection proceeds to the adjacent cell at pit fields (clusters of plasmodesmata). As the hyphae moves along the cell wall, it shrinks to a tapering diameter. Invasive hyphae comprises of a membrane cap consisting of membrane layer that secretes protein into the cytosol of the living host cell.

1.3.2 Components in PTI Recognition and Signalling

The fungal cell wall consists of chitin, glucan and other polymers that are cross-linked with each other. Chitin (β-1,4-linked N-acetylglucosamine) is recognized as a PAMP by plant PRRs (Adams 2004). Defence responses are triggered by several rice chitin PRRs as a result of the recognition of chitin fragments directly or indirectly (Kaku et al. 2006; Shimizu et al. 2010; Zeng et al. 2012). Chitin oligosaccharide elicitor-binding protein (CEBiP) is the utmost chitin-binding protein in rice cells. CEBiP is a RLP (receptor like protein) and shows very high affinity towards the polysaccharide (Kaku et al. 2006). It consists of a transmembrane domain and two LysM motifs but lacks an intracellular kinase domain. Thus another additional protein, OsCERK1 (chitin elicitor receptor kinase 1), is vital for the triggering of chitin-derived signals inside the cell. OsCERK1 is a RLK protein that consists of LysM domain and functions in co-ordination along with CEBiP in transducing the chitin-triggered rice immune response (Miya et al. 2007). It was reported that heterodimers of OsCERK1 and CEBiP were formed when the rice cells were treated with chitin oligosaccharides (Shimizu et al. 2010). In the prokaryotes, a LysM-containing protein (LYPs) binds to peptidoglycan (PGN), and in the eukaryotes (plants), it binds to the peptidoglycan-related chitin or Nod factors (Silipo et al. 2010). LYP4 and LYP6 are the chitin receptors that were identified in rice (Liu et al. 2012). Silencing of any of the abovementioned gene would result in reduced chitin-triggered immunity and may lead to compromised resistance against rice blast fungus.

1.3.3 Components in ETI Recognition and Signalling

There are certain specific molecules that are secreted by the pathogens into the plant cytoplasm interfering with plant defence mechanism known as effectors (Hogenhout et al. 2009). The cellular homologous R proteins recognize Avr (avirulence) in a direct or indirect manner, thereby triggering an effective hypersensitive response. A total of 15 effectors released by *M. oryzae* have been identified till date that includes 9 Avr effector proteins like PWL1, PWL2, AvrPi-ta, AvrPiz-t, Avr-Pia, AvrPii, Avr-Pik/km/kp, Avr1-CO39 and ACE1 and 6 newly characterized effector proteins

that includes 4 secreted biotrophy-associated secreted (BAS) proteins (BAS1, BAS2, BAS3, BAS4,), MC69 and Slp1. One of the Avr effectors, ACE1, encodes a hybrid polyketide synthase-nonribosomal peptide synthetase (PKS–NRPS), while the remaining eight effectors encode recognized secreted proteins without any cognate proteins in the databases (Liu et al. 2014). Slp1 suppresses rice PTI by interfering with rice chitin elicitor receptor protein CEBiP (Mentlak et al. 2012). MC69, a characterized protein consisting of a signal peptide, was identified to be a novel secreted protein and is required by rice blast fungus for establishing the infection. Knockout of *MC69* in *M. oryzae* fails to develop symptoms of the disease in rice plant. Also *MoHrip1* (*M. oryzae* hypersensitive response inducing protein 1) was distinguished by protein purification method. It is correlated with strings of defence responses such as production of hydrogen peroxide and deposition of callose and is responsible for inducing necrotic cell death in *Nicotiana benthamiana*. In rice PTI, MoHrip1 is also involved as it confers partial resistance to *M. oryzae* inducing the expression of some genes playing role in defence mechanism (Chen et al. 2012). But further investigation is required in rice to determine if the R protein will be able to recognize MoHrip1. Transcriptome analysis of rice leaves infected by blast fungus showed a total of 851 *in planta* genes were expressed that encodes the predicted effector proteins (Chen et al. 2013). The resistance mediated by the R genes during the co-evolutionary process of plant and pathogen interaction has helped in the development of abrupt and sturdy defence against the invading pathogen. Sharma et al. (2012) have reported that almost 100 R genes have been discovered up till now that confer resistance to *M. oryzae*. Also 21 R genes, namely, *Pib*, *Pita*, *Pi9*, *Pi2*, *Piz-t*, *Pi36*, *Pi37*, *Pikm*, *Pit*, *Pi5*, *Pid3*, *Pi54* (*Pikh*), *Pish*, *Pik*, *Pik-p*, *Pia*, *Pi25*, *Pb1*, *Pi1*, *Pi-d2* and *Pi21*, have been cloned leading to the characterized R genes. Most of the genes are the nucleotide-binding site leucine-rich repeat proteins (NBS–LRR) except for *Pi-d2* that encode RLK protein (Chen et al. 2006). Pi21 protein is rich in proline without any cognate in the databases (Fukuoka et al. 2009). The effector protein (Avr) and the R genes have been studied extensively. This includes *AvrPita* versus *Pita*, *Avr-Pik* versus *Pik*, *AvrPiz-t* versus *Piz-t*, *Avr-Pia* versus *Pia* and *Avr1-CO30* versus *Pi-CO39*. There is direct interaction between AvrPita and Pita and Avr-Pik and Pik in contrast to the indirect interaction between AvrPiz-t and Piz-t. Further the Avr-Pia and Avr1-CO39 interact with their homologous R proteins which are yet to be determined.

Figure 1 briefly explains the general mechanism of plant pathogen interaction where bacteria propagate especially in the extracellular regions of host tissues. Mostly fungus and oomycetes extends the hyphal region within this area and many forms haustoria. These structures penetrates only the cell walls of the plant but cannot go past the cellular membrane. The pathogenic elicitors also known as PAMPs are released into the extracellular regions. The PRRs recognizes the PAMPs resulting into the elicitation of PAMP-triggered immunity (PTI). The interaction of PRRs with the related protein Brassinosteroid insensitive 1-associated kinase 1 (BAK1) initiates the PTI signalling pathway. To counteract the PTI, the pathogens release the effector proteins into the plant cell. But in turn, intracellular

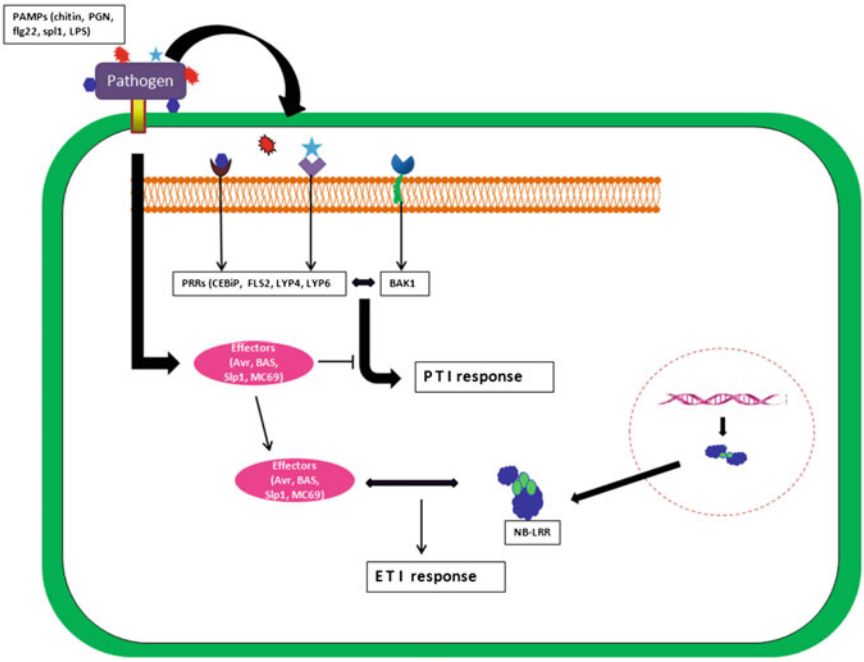

Fig. 1 General mechanism of host-pathogen interaction in plants

nucleotide-binding (NB)-LRR receptors recognize the effector molecules, thereby inducing effector-triggered immunity (ETI).

1.4 Functional Genomics in Rice

To apprehend the rapidly cumulating data and the functioning of the cell at global level, there is a need for high-throughput functional genomics. Functional genomics includes the study of how genes and intergenic regions of the genome contribute to different biological processes. Several genes or regions on a genome-wide scale (i.e. all or multiple genes/regions at the same time) are studied with the hope of further narrowing them down to a list of candidate genes or regions to analyse in more detail. Functional genomics focuses on the dynamic expression of gene products in a specific context, for example, at a specific developmental stage or during a disease.

Rice is regarded as a model plant species for functional genomics research because of the smaller genome size, precise genome sequences identified by co-linearity with the sequences of other important cereal crops (corn and wheat) and highly efficient transformation technology that has gained extensive advantage. Rice is the first genome crop plant that was sequenced with high precision. Most of the rice-producing regions have a long history and huge scale of genetic research,

Table 1 Summary of representative genomics databases for rice functional genomics

Database	Description	Web link
BGI-RIS	Genome annotation	http://rise2.genomics.org.cn/
DRTF	Rice transcription factors	http://drtf.cbi.pku.edu.cn
ECOGEMS	SNP database	https://github.com/venyao/ECOGEMS
HapRice	SNP haplotype database	http://qtaro.abr.affrc.go.jp/
IRRI	Germplasm and natural variation of rice	http://irri.org/
MCDRP	Rice proteins database	http://www.genomeindia.org/biocuration/
OGRO	Genome annotation	http://qtaro.abr.affrc.go.jp/ogro
OryGenesDB	Rice mutant database	http://orygenesdb.cirad.fr/
Oryzabase	Phenotype description and classification of rice	http://shigen.nig.ac.jp/rice/oryzabase/
Phospho Rice	Rice-specific phosphorylation sites	http://bioinformatics.fafu.edu.cn/PhosphoRice
POSTECH RISD	Rice mutant resource database	http://www.postech.ac.kr/life/pfg/risd/
RAP-DB	Genome annotation	http://rapdb.dna.affrc.go.jp/
QlicRice	Abiotic stress-responsive QTL	http://cabgrid.res.in/nabg/qlicrice.html
RGAP	Genome annotation	http://rice.plantbiology.msu.edu/
RICD	Rice indica cDNA database	http://202.127.18.221/ricd/index.html
RiceGE	Functional genomics express database	http://signal.salk.edu/RiceGE/RiceGE_Data_Source.html
RiceNet	Genome-scale gene network	http://www.inetbio.org/ricenet/
RiceVarMap	Rice genomics variation database	http://ricevarmap.ncpgr.cn/
RiceXPro	Rice expression profile database	http://ricexpro.dna.affrc.go.jp/
RIGW	Genome annotation	http://rice.hzau.edu.cn/rice/
RKD	Protein kinase	http://ricephylogenomics.ucdavis.edu/kinase/
ROAD	Rice expression profile database	http://www.ricearray.org/
RiceSRTFDB	Rice transcription factors	http://www.nipgr.res.in/RiceSRTFDB.html/

germplasm resources in abundance and large-scale breeding applications (Jiang et al. 2012). Once the whole genome of rice was sequenced, many functional genomics platforms have been constructed. This includes collecting the germplasm resources and generating the mutant libraries, cDNA libraries, microarrays, RNA-sequencing (RNA-seq), etc. (Yang et al. 2013). Gradually, the principles of proteomics, metabolomics, epigenomics and phenomics have also been established and improved, and parallelly platforms to analyse bioinformatics surveys and databases have also been set up in rice (Rajasundaram and Selbig 2016). Recently CRISPR-Cas9/Cpf1 gene editing techniques have become a powerful tool for gene mutagenesis in rice functional genomics research (Feng et al. 2013). Table 1 lists some of the representative genomics databases for rice functional genomics which are freely available to the rice research community.

2 Role of Genomics in Understanding the Mechanism of Host-Pathogen Interaction

Genomics approaches/techniques are major player in plant biology to study biomolecules (DNA, RNA, protein and the metabolites). Nowadays, with the advent of high-throughput techniques, determination of function of a gene is quite easy as compared to conventional techniques. The biotic stress is one of the major constraints for crop productivity, and omics tech assists in understanding of changes in plant due to biotic stressor, thereby helping plant biologist to breed/engineer crops to cope with biotic stress (Dong and Ronald 2019). Rice is rich in biodiversity, and IRRI International Rice Gene bank (IRG) has largest collection of rice germplasm (Jackson 1997). These varieties have diverse characteristic, and among them some varieties have extraordinary trait like disease resistance from pathogen, better performance in certain condition, etc. With completion of hundreds of rice germplasm genome sequencing, availability of T-DNA and transposon insertion line and small genome, rice acts as a model for monocot/cereal crop (Li et al. 2018).

An overview of the approach that can be employed to identify the genes affecting the biotic stress tolerance and engineering tolerance in plant is shown in Fig. 2. These genomics-based approaches assist in identification of gene associated with the biotic stress which can provide either tolerance or susceptibility to disease; accordingly these genes are used to introduce tolerance in disease susceptible plant by overexpression/introgression or downregulation/knockout, respectively. Many R genes which confer resistance to disease in rice were introgressed in susceptible cultivar (Ansari et al. 2015). *Os11N3* (also called *OsSWEET14*) is a rice bacterial blight susceptibility gene that encodes a member of the SWEET sucrose-efflux transporter family. The gene is modulated by *Xanthomonas* through its TAL (transcription activator like) protein so that more sugar is transported in extracellular space that makes better environment for Xoo survival. These families of proteins have been targeted through genome editing tools like TALEN and CRISPR/Cas9 system to resist with disease (Li et al. 2012; Zhou et al. 2014). Along with these approaches, new HTS-based strategies are being employed to identify new R genes and decipher the mechanism of host-pathogen interaction like RenSeq, iPOOL-seq and chimeric sequence. To broaden the R gene diversity, a CRISPR/Cas9-nickase-based mutagenesis strategies are developed known as EvolvR for directed mutagenesis of in specified target window (of DNA) to diversify R gene recognition and activity. This EvolvR approach can be used to engineer Xa21 gene of rice in such a way that it can recognize broad array of *Xanthomonas* strain (Pruitt et al. 2015). A genome-scale protein-protein interaction tools, RiceNet, have been developed that can accurately predict protein partners, and this can be also utilized for other monocotyledons. Using RiceNet, two positive regulators and one negative regulator that interact with XA21 have been identified and validated in planta (Lee et al. 2011). These emerging genomic technologies will help to decipher genes function and involvement in biotic stress using the genomic resources of rice.

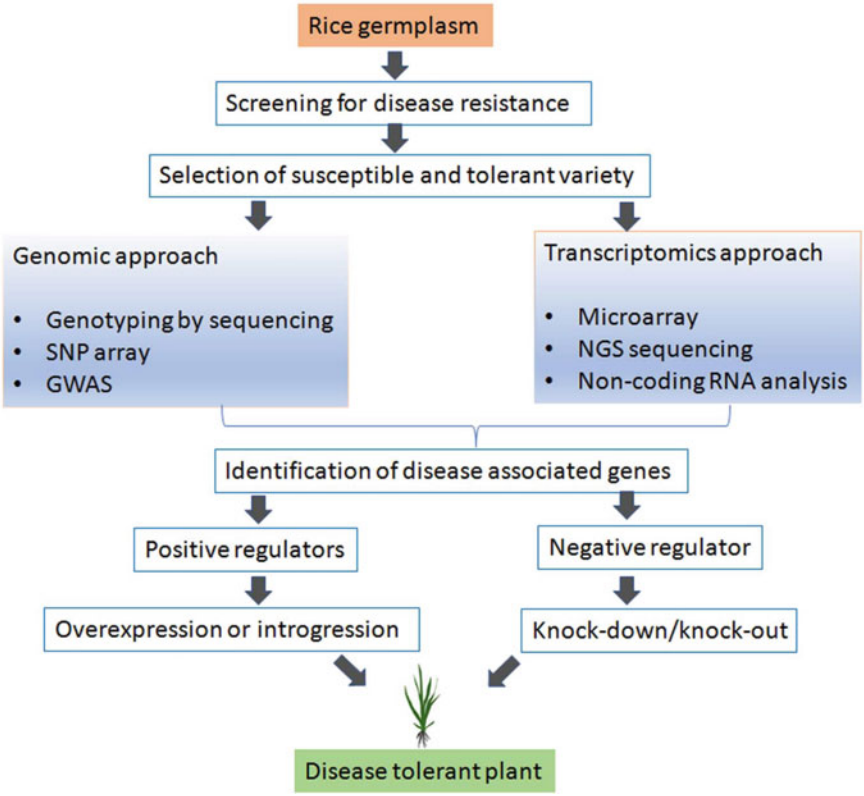

Fig. 2 Genomics approaches for identification of stress associated gene and introduction of stress tolerance in susceptible plant lines

3 Genome-Wide Association Study (GWAS) for Developing Disease Resistance in Rice OR GWAS to Understand Mechanism of Host-Pathogen Interaction in Rice

Genomes vary between individuals in different ways, and the differences are in the DNA sequences. These differences can be due to SNPs (single nucleotide polymorphisms), deletions or insertions of the bases in the genome. With the sturdy development of high-throughput sequencing technologies, GWAS (genome-wide association study) is a new tool for examining important agronomic or disease-related traits of rice (Fig. 3). GWAS performs association studies using genetically highly diverse germplasms between agronomically important traits and whole-genome genotypes. Recently, GWAS has started gaining popularity in rice genetics and is being used in amalgamation with NGS like genotyping by sequencing (GBS) that identifies the SNP markers linked with phenotypes that are resistant in rice

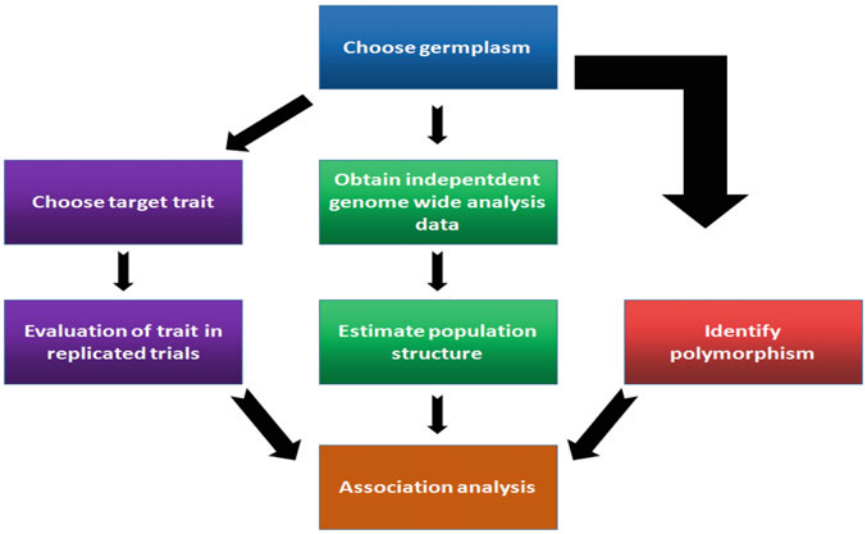

Fig. 3 A schematic representation of genome-wide association study (GWAS)

cultivars (Abe et al. 2012; Yu et al. 2014). To gain a glimpse of the disease resistance in rice, we have summarized the representative genes in Table 2.

3.1 Association Mapping of R Genes in Rice

Association mapping method maps quantitative trait loci (QTL) that takes advantage of linkage disequilibrium in linking phenotypic to genotypic traits. Hence it is also known as linkage disequilibrium mapping. The basis of the mapping process is that the location of the marker locus and the trait locus are close enough so that during recombination process the marker allele will associate along with the trait allele in several generations (Rodriguez-Murillo and Greenberg 2008). The focus of this method is identification of genetic variants among the individuals, like single nucleotide polymorphisms (SNPs), that shows the strongest linkage with the phenotype of interest. The reason behind such association could likely be due to the statistical correlation or linkage disequilibrium (LD) with an unobserved causal variant.

In the past decade, a range of association mapping studies have been conducted for rice cultivars of which recent are being mentioned briefly. Dilla-Ermita et al. (2017) reported a large-scale GWAS, genome-wide association study of bacterial blight resistance. A variation of 285 rice accessions was selected to locate the loci that show association with resistance to nine Xoo strains from Philippines. Strong connections were discovered for novel SNPs that showed linkage with familiar bacterial blight resistance genes, i.e. Xa genes. Such findings led to designing of high utility markers to track and select the genes responsible for resistance in

Table 2 Representative genes related to disease-resistant traits in rice

Gene	Accession no.	Pathogen	Disease	Annotation description	References
Bsr-d1	LOC_Os03g32230	*Magnaporthe oryzae*	Rice blast	ZFN transcription factor	Li et al. (2017b)
COPT1	LOC_Os01g56420	*Xanthomonas oryzae*	Bacterial blight	Copper transporter gene	Yuan et al. (2010)
COPT5	LOC_Os05g35050	*Xanthomonas oryzae*	Bacterial blight	Copper transporter gene	Yuan et al. (2010)
GH3-8	LOC_Os07g40290	*Xanthomonas oryzae*	Bacterial blight	IAA amino acid synthetase	Ding et al. (2008)
IPA1	Os08g0509600	*Magnaporthe oryzae*	Rice blast	Encodes OsSPL14 (SQUAMOSA promoter binding protein-like 14) transcription factor	Wang et al. (2018)
LHS1	LOC_Os03g11614	*Magnaporthe oryzae*	Rice blast	MADS-box gene	Yi et al. (2009)
OsBBI1	LOC_Os06g03580	*Magnaporthe oryzae*	Rice blast	RING-type E3 ubiquitin ligase	Li et al. (2011)
OsRac1	LOC_Os01g12900	*Magnaporthe oryzae*	Rice blast	Small GTP-binding protein	Nakashima et al. (2008)
Pi21	LOC_Os04g32850	*Magnaporthe oryzae*	Rice blast	Proline-rich protein	Fukuoka et al. (2009)
OsPAL4	LOC_Os02g41680	*Magnaporthe oryzae*	Rice blast	Phenylalanine ammonia-lyase gene family	Tonnessen et al. (2015)
PigmR/S	KU904633	*Magnaporthe oryzae*	Rice blast	*Pyricularia oryzae* resistance gm	Deng et al. (2017)
Rac1-RbohB/H	LOC_Os01g25820	*Magnaporthe oryzae*	Rice blast	Respiratory burst oxidase homolog	Nagano et al. (2016)
RACK1	LOC_Os01g49290	*Magnaporthe oryzae*	Rice blast	Receptor for activated C kinase 1	Nakashima et al. (2008)
STV11	LOC_Os11g30910	Rice stripe virus (RSV)	Viral disease of rice	Sulfotransferase	Wang et al. (2014b)
TIG1	EDK00306	*Magnaporthe oryzae*	Rice blast	HDAC transcriptional corepressor complex	Ding et al. (2010)
Tps1	LOC_Os08g34580	*Magnaporthe oryzae*	Rice blast	Trehalose-6-phosphate synthase gene	Wilson et al. (2010)

WRKY13	LOC_Os01g54600	*Rhizoctonia solani* and *Sarocladium oryzae*	Sheath blight & Sheath rot	Transcription factor in plant signalling pathway	John Lilly and Subramanian (2019)
Xa10	LOC_Os11g37620	*Xanthomonas oryzae*	Bacterial blight	TAL effector-dependent R gene	Tian et al. (2014)
Xa21	LOC_Os11g35500	*Xanthomonas oryzae*	Bacterial blight	Receptor kinase-like protein	Park and Ronald (2012)
Xa25	LOC_Os12g29220	*Xanthomonas oryzae*	Bacterial blight	MtN3/saliva family protein	Richter et al. (2014)
XB24	LOC_Os01g56470	*Xanthomonas oryzae*	Bacterial blight	ATPase	Chen et al. (2010)

breeding approaches. Further it was found that the SNPs identified in chromosomes 6, 9, 11 and 12 are significantly associated, and no overlapping was observed with existing resistance loci revealing it be novel sources of resistance. Comprehensive analysis presented an outline regarding the haplotypes that corresponds with resistance. Further analysis of presumed resistance alleles leads to identification of resistant genotypes that can be the prospective donors of new genes responsible for resistivity.

Zhang et al. (2017) conducted a GWAS and confirmed two hotspot regions associated with Xoo resistance on chromosomes 11 and 12. The region that was identified on chromosomes 11 and 12 included 89.6% and 85.3% of the SNPs significantly associated with blight resistance, respectively. Numerous retrotransposons and transposons were also confirmed among the candidate genes consisting of the SNPs related with the disease resistance. Thus it can be summarized that during the evolutionary process, transposable elements have refined the diversity of bacterial blight resistance genes. Further 12 bacterial blight resistance loci were also identified.

Another report on GWAS was given by Kang et al. (2016) where the rice diversity panel 1 (RDP1) was genotyped using 700,000 SNPs array identifying 97 loci related with blast resistance of which 15 were familiar loci and the remaining 82 were confirmed to be new regions. They combined the GWAS with RNAi (RNA interference) mechanism that was successful to identify the resistant allelic regions at the Pi5 locus. Hence this illustrated that the combination of both the technologies proved to be efficient for the investigation of alleles resistant to complex diseases such as rice blast.

A total of 30 loci were identified in the indica panel that showed association with rice blast disease resistance. Some association was also reported on the third chromosome in contrast to the earlier reports that mentioned no association of loci with rice blast resistance on chromosome 3 (Wang et al. 2014a). Thus several reports in indica cultivar give an overview that GWAS is indeed a powerful technology and immensely effective to study association mapping.

3.2 QTL Mapping in Rice

Quantitative trait loci (QTL) mapping was developed during the late 1980s for identification of genes or QTLs responsible for quantitative traits. It was a milestone in the field of plant genetics (Doerge 2002; Semagn et al. 2010). Since then, there have been many reports of genes or QTLs linked with significant traits ranging between various plant species.

As already mentioned in the previous section, the significance of rice blast fungus and the effects of the disease in rice production at an alarming rate have led to the mapping of around 100 QTLs resistant against the disease. The QTLs are dispersed on all 11 chromosomes; chromosome 3 is an exception. QTLs are more dense on chromosomes 6, 11 and 12. Apart from that, 27 genes, namely, Pib, Pi-ta, Pi9, Pi2, Piz-t, Pi-d2, Pi33, Pii, Pi36, Pi37, Pikm, Pit, Pi5, Pid3, Pid3-A4, Pi54, Pish, Pik,

Pik-p, Pi-CO39, Pi25, Pi1, Pb1, Pi64, LABR_64-1, LABR_64-2 and Pigm, have been cloned so far. Interestingly the R (resistance) genes belong to NBS-LRR (nucleotide binding site and leucine-rich repeat) family. Pigm consists of numerous NBS-LRR clustered genes. The functional protein, viz. PigmR, is associated with broad-spectrum resistivity, and PigmS competes with PigmR attenuating the formation of PigmR homo-dimers so that the resistance is suppressed (Deng et al. 2017). The bsr-d1 gene promoter when subjected to a single nucleotide change accords to blast resistance in a wide range (Li et al. 2017a).

Bacterial blight is another serious disease in rice caused by *Xanthomonas oryzae*. It can affect the yield up to 70% when the susceptible varieties are cultivated. Hence managing the disease by using the resistant variety is till now the most effective and reliable method. Accounting the seriousness of the disease, 40 QTLs for bacterial blight resistance have been reported. Altogether, 11 genes have been cloned until now that include 7 dominant genes, viz. Xa1, Xa3/Xa26, Xa4, Xa10, Xa21, Xa23 and Xa27, and 4 recessive genes, viz. xa5, xa13, xa25 and xa41 (Zhang and Wang 2013). Some recent blight disease resistance genes are mentioned in Table 2. The function of the proteins encoded by the abovementioned genes is briefly discussed further. Xa1 belongs to NBS-LRR family of disease resistance genes in plant (Yoshimura et al. 1998). Xa23, xa13, xa25 and xa41 code for a transmembrane protein (Cao et al. 2018; Zhang et al. 2017; Wang et al. 2015). Xa3/Xa26 and Xa21 code for LRR-kinase proteins; xa5 provides race-specific resistance to *X. oryzae* pv. *oryzae* and encodes the small subunit of transcription factor IIA (Huang et al. 2016). Xa4 codes for a cell wall-associated kinase that facilitates cell wall strengthening and promotes synthesis of cellulose and suppresses cell wall loosening (Hu et al. 2017).

A very brief mention about the viral diseases in rice includes rice stripe virus that destructs rice yield mainly in japonica cultivars and is prevalent in China, Japan and Korea. In indica cultivar reports have mentioned that 5 QTLs for stripe virus resistance are located on chromosome 11. This includes Stv-bi, qSTV11IR24, qSTV11TQ, qSTV11KAS and qSTV11SG. OsSOT1, a sulfotransferase encoded by STV11-R, converts the salicylic acid (SA) into sulfonated SA (SSA) (Wang et al. 2014b).

3.3 Molecular Markers (SSR and Others) to Identify Disease Resistance Association in Rice

Molecular markers have played an extensive powerful role in rice breeding and genetic studies. Microsatellites are the most commonly used molecular markers because they can be easily amplified by PCR, and at each locus, the huge amount of variation in allele can be observed. Microsatellites include one to six repeats of nucleotides; hence they are also known as simple sequence repeats (SSR). These molecular markers are found in abundance, dispersed throughout the genomic regions, and show high polymorphism in comparison to the other markers. They are species-specific and possess co-dominance. Hence amidst the numerous existing

molecular markers, these markers have evolved as the choice markers and are significantly important in rice breeding applications. Among the more noteworthy examples of genes that have been associated to genetic markers in rice are those that provide resistance or tolerance to rice blast disease (Gupta and Varshney 2000). Two RAPD markers linked to the Pi-10t locus were identified against rice blast disease in Korean rice variety. Andargie et al. (2018) reported that 176 rice plants were used to demonstrate QTL mapping for resistance against rice false smut disease. Altogether 360 simple sequence repeat (SSR) markers were screened to check polymorphism on the parents, susceptible and resistant bulks, that yielded SSR markers associated to gene resistant against the disease. Further the bulk segregant was analysed that located the position of the resistance gene to chromosome 5. Also on the same chromosome, QTL analysis revealed two QTLs that gave the phenotypic variations of 7.3% and 16.4%.

3.4 SNP Chip Array Techniques for Disease Resistance in Rice

Conventional molecular marker techniques, such as RFLP (restriction fragment length polymorphism) and SSR (simple sequence repeat), play a significant role in the research on functional genome. However, they have many limitations such as low throughout, low quantity and complicated operation processes, and they do not meet the needs of large-scale breeding for commercial purpose. At present, there are mainly two platforms for high-throughput molecular marker techniques; one is based on the second-generation sequencing technology, and the other is based on gene chip technology. Molecular marker techniques based on gene chip mainly include SNP array. In the study of genetics, SNPs are basically used as *markers* of a region on the genome, where due to its occurrence there is hardly any effect on the ongoing mechanisms on the biological system. But SNPs also can have physiological consequences, such as variation in single amino acid, will cause changes to stable mRNA transcript and can result in changing of the binding affinity of the transcription factor (Griffith et al. 2008). The single nucleotide polymorphism (SNP) microarrays were invented almost 20 years ago. Since then the technology has developed and screenings have been made more faster, with more extension and reduced economical cost, SNP markers show co-dominance and are biallelic which are in abundance and uniformly distributed throughout the genome (Mammadov et al. 2012). Thus these molecular markers are reliable in the development of a high-density genetic map. A high-density single nucleotide polymorphism (SNP) array in rice consisting of 51,478 markers was developed on the Illumina Infinium platform that can be widely used for functional genomics approaches and rice molecular breeding (Chen et al. 2014).

Rice Whole-Genome Breeding Chip Several medium- or high-resolution SNP arrays have been established in rice. This includes a 44 K SNP chip (Zhao et al. 2011), 50 K SNP chips (Singh et al. 2015) and the 700 K high-density rice array (HDRA) (McCouch et al. 2016). These breeding chips are mainly used for GWAS.

These arrays allocate platforms that are automatic and dissect phenotype-genotype correlation simultaneously offering datasets used for validation of SNP markers of high quality that have information within and between key germplasm categories. The rice whole-genome breeding chip of the present invention is Rice 60 K, an SNP chip manufactured based on Infinium technique.

Genomic Selection Genomic selection is a type of new breeding method. It utilizes the genome-wide DNA marker data to ameliorate the breeding efficiency for quantitative characteristics. Spindel and Iwata (2018) gave reports of identifying and selecting the individuals of a breeding population that has high-quality breeding values on the basis of the prediction models that was built to find the correlation between phenotype and genotype.

Genomics-Assisted Breeding In the past few years, genomics-assisted breeding (GAB) has brought an impactful change in plant breeding. GAB as the name suggests assists in breeding practices by integrating the genomic tools with high-throughput phenotyping using DNA markers that predict phenotype from genotype (Leng et al. 2017). It is a very advantageous method as it is highly accurate, direct improvement, short-term breeding cycle and highly selection efficient. Therefore GAB is especially remarkable for the improving of complex traits. The main aim of GAB is to locate the ideal combinations of alleles or haplotypes, optimum gene networking and definite or particular regions in the genome to promote crop production (Xu et al. 2012). In addition, GAB promises to expedite the generation of new plant cultivars and improve the growth of modern agriculture.

3.5 Genotyping by Sequencing (GBS)

Various chip-based techniques and single nucleotide polymorphism (SNP) screening are the methods of choice in scrutinizing and associating traits with genomic regions for many plants and animals. With the decrease in the sequencing cost, new approaches are being developed that leverage next-generation sequencing (NGS) for genotyping that has been the game changer for genomics and genetic studies. Genotyping by sequencing is also known as next-generation genotyping. It screens the genome to identify and discover the novel SNPs in plants and animals so as to further perform the genotype studies. A study was conducted where GBS-based genetic diversity population analysis when combined with association studies was found to be a powerful approach in characterizing R genes/QTLs against rice blast disease that promotes resistance against *M. oryzae* belonging to African population. Markers linked to RABRs (regions associated with blast resistance) and 14 highly resistant cultivars against rice blast were identified in this study (Mgonja et al. 2016).

3.6 Genome-Wide Meta-analysis and Phenomics for Disease Resistance Genes

In meta-analysis, combined data from various sources in bulk quantity are analysed in a single study. This approach is utilized in different fields of sciences like medical, social and behavioural sciences. Meta-analysis acquires QTL from various populations and uses different traits to measure resistance, thereby allowing to estimate the statistics of some QTLs if in fact they actually complement to one single QTL; thus it gives an estimate of the location of this metaQTL (Goffinet and Gerber 2000). The meta-analysis study was conducted for rice blast-resistant QTL that detected 165 meta-QTL; thus the initial dataset of 347 QTL was remarkably reduced. It was reported that overall a total of 347 rice blast resistance QTLs over the 12 chromosomes have been mapped. Thus for further analysis on such a huge QTL number and in order to refine their positions, the information was summarized (Ballini et al. 2008).

4 Advanced NGS Tools for Host-Pathogen Interaction Studies in Rice

4.1 Next-Generation Sequencing

In post-genomics era, the pace in understanding the mechanism of host plant and pathogen interaction has undergone with a speedy rate, and scientists are chronically on the verge to decipher genome information such as structure and functions of both plants and pathogens to come out for the global solution of crop improvements ensuring food security with plants having a broad spectrum of resistance to multiple biotic stresses (Imam et al. 2016). Emergence of omics technologies such as metagenomics, transcriptomics and proteomics has help in understanding the stored genome information in plants in contact with pathogens of known and unknown species and depth knowledge into the molecular changes that is occurring. Sequencing technologies played a major backbone in the utilization of these omics approaches (Zhang and Li 2016). Next-generation sequencing contributes to the generation of large sequence data of an organism genome within a short time period and at relatively low cost, and it being widely adopted compared to Sanger first-generation sequencing because of its ability to massively sequence nucleotide bases of millions of fragments at a time generating to up to gigabase of data (Kulski 2016). High demand for NGS sequencing has led many companies to develop new and more advance form of the technology giving better performance and has gone from second-generation sequencing machines (including Roche 454 pyrosequencing, Illumina/Solexa, SoLiD and Ion Torrent) characterized by the needs to prepare sequencing libraries to the next level by sequencing single DNA molecules and direct RNA-sequencing called the third-generation sequencing (PacBio/SMRT, Heliscope and Oxford Nanopore) (Yadav et al. 2016). Sequence data are stored for public availability in NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/

sra). Combination of these technologies can be used to improve assembled genome quality for sequencing plants genome (Mahesh et al. 2016).

Performing NGS in rice is unavoidable demanded because of its well-sequenced high-quality genome and annotations, besides being an important model crop among the monocot plant species (Jackson 2016). Using high-throughput NGS technology can identify candidate genes that have the potential to improve plant beneficial traits such as grain width, plant height, disease resistance, etc. (Silva et al. 2012). Understanding the complex information on the performance of genes with respect to particular plant traits required the investment of high-throughput and reliable computational bioinformatics and algorithm tools to translate NGS data into an understandable formats (Sikhakhane et al. 2016). NGS has contributed as a powerful tool in the identification of genetic variations in both the pathogens and host plant such as InDels and single nucleotide polymorphisms (SNPs) that led to the discovery of potential plant pathogens virulence factors, pathogenicity-related genes and understanding plants gene functions as a whole (Cao et al. 2017). In plant genetics SNPs have wide application in studies such as linkage mapping, genome-wide association mapping studies, evolutionary studies, population structure, marker-assisted plant breeding and functional genomics (Kumar et al. 2012). Thousands to millions of SNPs can be identified by undergoing whole-genome re-sequencing and comparing sequence genome between same species and to a reliable reference genome, as in the case studied by Xu et al. (2017), where two wild rice genotypes were re-sequence to explore SNPs and naturally occurring broad-spectrum blast resistance gene nucleotide-binding site leucine-rich repeat (NBS-LRR) encoding genes (Liu et al. 2013) and other novel resistance genes that have the potential in breeding for cultivated rice. Whole-genome sequencing has led to the identification of non-synonymous SNPs comparing two different rice inbred lines resistant and susceptible conferring resistance to sheath blight disease (Silva et al. 2012). The identification of quantitative trait loci (QTLs) and their associated marker controlling plant traits of interest has been extensively studied for plant breeding purposes (Tsaneva et al. 2019) using traditional method; however its application is restricted by its time consumed and high cost. Takagi et al. (2013) undergo whole-genome re-sequencing of DNA what is called as QTL-seq in combination with bulked segregant analysis to identify QTLs from population of rice recombinant inbred lines and F_2 generation by crossing a partially resistance cultivar with a highly susceptible cultivar and successfully identify those that confer partial resistance to fungal blast disease and seedling vigour. In due course of evolution, genetic variability among many fungal or bacterial isolates tends to occur leading to their adoption of novel potential pathogenicity factors that caused plant disease control a major issue. NGS technology has help in identifying these genetic changes that are occurring in the field through various next-generation sequencing platforms. A comparative analysis study was conducted where field isolates of *Magnaporthe oryzae* (B157 and MG01) were collected from different regions of southern India to understand genomics variations and isolate specific gene content that includes findings in which MG01 contains more host specificity factors and Avr genes as compared to B157, identification of SNPs, repeat elements in both isolates and their

insertional mutagenesis in Avr genes that leads to the emergence of new virulent strains (Gowda et al. 2015). In plant breeding perspective NGS played a pivotal role in the mining of genetic variations using method such as genotyping by sequencing and whole-genome re-sequencing that led to the development of useful molecular markers (Vlk and Řepková 2017).

Plants reprogrammed their gene expressions when in contact with pathogens and developed immunity for its survival. Their immunity responses triggered the activation of defence pathways: pathogen-triggered immunity (PTI) and effector-triggered immunity (ETI) leading to hypersensitive response and reduction in oxidative stresses, as well as activation of signalling pathway genes and hormonal crosstalk (Jones and Dangl 2006). Energy-storing macromolecules such as lipids and starch have also been reported to take part in plant signalling pathway for biotic stress tolerance (Tian et al. 2018). RNA-sequencing or transcriptomics profiling has help in capturing these transcriptional changes undergoing in both plants and pathogens leading to their compatible or incompatible reactions, getting valued information for crop improvement (Qi et al. 2018). RNA-sequencing has unravel many rice genes involved in defence mechanisms of disease such as blast *M. oryzae* infecting both aerial leaves and roots of rice plant (Tian et al. 2018), bacterial blight *X. oryzae* (Tariq et al. 2018), fungal sheath blight *Rhizoctonia solani* (Kouzai et al. 2018), false smut disease *Ustilaginoidea virens* (Han et al. 2015) and seed-borne disease *Fusarium fujikuroi* (Matić et al. 2016). Genome-wide transcriptomics profiling of rice susceptible to virus pathogen *Rice stripe virus* reveals photosynthetic and flowering genes negatively impacted by the virus, whereas genes involved in metabolic pathways, stress response and transcription show defence mechanism for the host (Wong et al. 2015). For better understanding of transcriptional profile in rice encountering a pathogen, gene co-expression network analysis can be done interpreting the regulatory function of genes that control a distinct phenotype (Zhang et al. 2018). Introduction of a disease resistance gene into plant is another alternative approach to study function of a gene such as in the case where rice isogenic lines 9311/Xa21 conferring resistance to bacterial blight disease was confirmed by transcriptomics profiling unveiling the primed role of Xa21 gene in disease resistance (Peng et al. 2015). Colonization and infection strategy of a mutualistic endophyte *Harpophora oryzae* and a pathogenic rice pathogen *Magnaporthe oryzae* leading to their contrasting responses have been studied by Xu et al. (2015) by undergoing transcriptional profiling and understanding the changes occurred with respect to the photosynthetic metabolites exchange, evolutionary changes of *H. oryzae*, their competitive behaviour, etc. that have future scope in employment biocontrol agent in controlling disease. Rice Expression Database (RED) is a user friendly database that has been created in which high-quality transcripts derived from different RNA-seq experiment is integrated that allow users to characterized housekeeping and tissue specific genes, studying gene co-expression network with an interactive genome browser visualization at a genome-wide scale (Xia et al. 2017).

As seen many of the studies interpret and have proven the functionality of rice and pathogen gene expressional changes individually; however clear concepts of their interaction that leads to disease causing stage can be attain when looking at the mechanism of their response simultaneously. This has led to the consideration of an RNA-seq technology called dual RNA-seq, or in planta RNA-seq, or simultaneous RNA-seq, or comparative RNA-seq where it allows the simultaneous sequencing of two species at the same time understanding their interactive mechanism (Kovalchuk et al. 2019). The application of this technology, a mixed transcriptome study, was carried out between *Oryza sativa* L. ssp. japonica cv. Nipponbare and *Magnaporthe oryzae* compatible and incompatible isolates to compare transcript changes at the initial infection stage. Rice defence-responsive genes such as PR proteins, phytoalexins, MAPK kinase genes and transcription factors WRKY and OSNAC family were found to accumulate abundantly in incompatible interaction as compared to compatible reactions and indicate the effective defence mechanism of rice against the incompatible isolates. Fungal transcripts playing role in initial plant infection were detected to accumulate for both the isolates. Genes involved in appressorium development, effector secretory proteins, LysM effectors which suppress PTI immunity, cutinase and several glycosyl hydrolase enzymes were specifically highlighted (Kawahara et al. 2012). This method has been fascinatingly applied in plant systems especially interacting with pathogens (Kovalchuk et al. 2019) and with the advancement of sequencing technology and bioinformatics tools has made the availability of many species reference genomes to easily separate mixed RNA species at the level of in silico platform. However essential steps and proper planning of a particular experiment are important to elucidate valid interactions (Naidoo et al. 2018), and the challenges and solutions inferred for non-model species have been described by O'Keefee and D. Jones (2018).

The role of non-coding miRNA in resistance against diseases has been reported for many plant species (Chow and Ng 2017) including rice (Baldrich et al. 2015), and rice pathogen *Magnaporthe oryzae* miRNA has been found to suppress immune defence response (Zhang et al. 2018). Through deep sequencing method, Baldrich et al. (2015) identify rice target genes subjected to miRNA regulation in response to *Magnaporthe oryzae elicitor* such as oxidative stress protective genes, transcriptional factors, defence genes such as RPP13 and MLO, defensin, PPR proteins, hormonal signalling, crosstalk-mediated genes, etc. and novel regulation of translational peptide sequence *CPuORF*, all have functional role in regulating rice response to the disease. Similarly Li et al. (2019) identified rice miRNA target *OsGRFs* (growth regulating factor) through RNA-sequencing possessing resistance to bacterial pathogen *Dickeya zeae* an emerging disease causing rice foot rot. The resistance mechanism against this particular disease in a resistant rice variety was used to investigate the participation of long non-coding RNAs (lncRNAs) using RNA-seq technology and identify potential transcriptional target genes as well as miRNAs playing key role in defence mechanism. One target example is the *osa-miR156* whose targets include ubiquitin protein ligase and glycosyltransferase having functional role in conferring resistance to rice pathogens (Li et al. 2018). A scheme

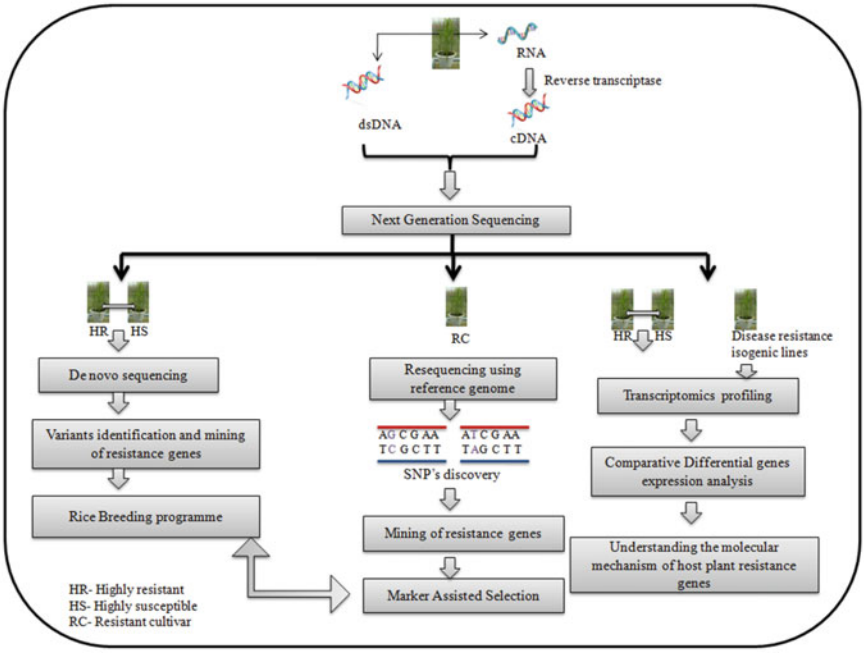

Fig. 4 Application of next-generation sequencing for functional genomics studies in rice response to biotic stress

representing the application of next-generation sequencing for functional genomics studies in rice response to biotic stress is given in Fig. 4.

4.2 In Vivo Insertion Pool Sequencing (iPOOL) for Identification of Virulence Factors in Rice

Identification of virulence factors in phytopathogens is important for developing an effective defence mechanism against the pathogen by the host. However individually assessing of fungal mutant gene in the host via insertional mutagenesis to study its function is a handful of laborious work and expensive (Michielse et al. 2009). Genome-wide identification of a pool of mutagenized insertion cassettes has been limited to few studies supported with the use of sequencing technologies such as randomly barcoded transposon mutagenesis sequencing (RB-TnSeq) used for mapping of microbial genes of bacterium *Pseudomonas simiae* strain WCS417r colonizing roots of *Arabidopsis* plant, thus understanding genes playing a positive and negative role in plant microbe association (Dong et al. 2017), the quantitative insertion-site sequencing (QIseq) used for the identification of insertion sites generated by piggyBac transposons in eukaryotic organism *Plasmodium falciparum* (Bronner et al. 2016) and is one of the few studies conducted to identify virulence

genes in eukaryotic pathogen infecting host because of the complex nature of host-pathogen interaction. It was Heitman et al. (2018) that first introduced a method called insertion pool-sequencing (iPool-seq) on maize infected with a pool of 195 mutants fungal pathogen *Ustilago maydis* assessing individuals function in the same host to enable successful quantitative identification of insertion mutant screen for known and novel virulence factors responsible for the disease. Its modelling serves as a versatile high-throughput technology for screening of mutants developed through knock-in, activation tagging or transposon insertion libraries reducing cost for understanding phenotype-genotype relationship.

5 Epigenomics in Gene Expression Reprogramming During Abiotic Stress in Rice

Epigenetics can be defined as study of difference in expression of a character due to modification of histone protein or modification of base (not sequence), i.e. at chromatin level. These changes may or may not be heritable mitotically and meiotically. Most common changes that are seen in organisms are post-translational modification of histone protein mainly methylation and acetylation along with phosphorylation, sumoylation and ubiquitinylation. The expression level of genes is dictated by type of epigenetic modifications (Pikaard and Mittelsten Scheid 2014). Acetylation of histone leads to activation of expression of gene, but methylation has both effect, i.e. activation and repression of genes that depend on the position and combination of histone code. In eukaryotes, mostly methylation of C base occurs at five position, and A is rarely modified (modification of A is common in bacteria) base in DNA. These changes either increase or decrease the expression of gene (Vidalis et al. 2016). In plant, epigenetic mechanism is known to affect germination, development, flowering and stresses, so deciphering the type of epigenetic changes in rice could lead to the crop improvement (Shi et al. 2015). In *Arabidopsis*, the epigenetic mechanism is best known compared to rice specially the regulation of flowering via FWA gene (Soppe et al. 2000). The rice genome on average more methylated (24.7%) than *Arabidopsis* genome (10.5%). In general, plant DNA methylation occurs at CG, CHG and CHH sites (H- A, C or T) (Köhler and Springer 2017). The frequencies of methylation in rice at CG, CHG and CHH individual sequence level are 44.6%, 20.14% and 4.02%, respectively, as compared to *Arabidopsis* with 30%, 14% and 6%, respectively (Li et al. 2018).

Plant can utilize epigenetic phenomenon to deal with stress, but few epigenetic studies have been reported for biotic stress as compared to abiotic stress that may be due to scientist still are not able to devise suitable strategies to study epigenetic regulation in biotic stress (Alonso et al. 2019). Upon interaction of plant with pathogen, pathogen can cause changes in epigenome of plant, and the epigenome in turn influences the pathogenicity of the pathogen (Latzel et al. 2013). The change in epigenome can activate the disease resistance gene in plant, for example, in rice expression of disease resistance gene in response to bacterial blight pathogen (*X. oryzae*) was increased by induced expression of histone lysine demethylase

(Li et al. 2013) and similarly treatment with DNA demethylating agent (5-azadeoxycytidine) also increased resistance to blight (Akimoto et al. 2007). In stress condition, to avoid unfavourable condition, plants flower early and produce seeds. The seeds can also transfer there epigenetic memory to future progenies; this phenomenon is known as transgenerational memory (Yaish et al. 2011). These epigenetic changes may lead to mechanistic basis to species survival and evolution of plant to adapt stress condition thereby plant offspring's can respond with future recurring stress effectively (Lämke and Bäurle 2017).

6 Other Functional Genomic Tools

Small RNAs (sRNAs) are short, non-coding RNA molecules that range in length from 20 to 40 nucleotides and guide gene silencing in most eukaryotic organism (Yu et al. 2017) at gene expression level: transcription or post-transcription (Iwakawa and Tomari 2015). In plants, sRNAs are divided into two main groups, microRNAs (miRNAs) and small interfering RNAs (siRNAs). They are differentiated by their mode of biogenesis and mechanisms of action (Niu et al. 2015). During the past decade, the sRNAs involved in plant responses to biotic stresses have gained more attention. Several reports in various plant species have illustrated the vital role of sRNAs and following are some briefly mentioned in biotic stress.

6.1 MicroRNAs Involved in the Regulation of Immunity in Rice

During the bacterial attack, the acquisition of the miR393 (first identified miRNA) in *Arabidopsis* is followed by apprehension of flg22 leading to negatively regulated transcripts for F-box auxin receptors and repression of auxin signalling. This in turn provides an increased resistance to bacteria, *Pseudomonas syringae* (Navarro et al. 2006). In case of a fungal encounter, miRNAs such as miR160a and miR398b are associated in modifying the immune response against *M. oryzae*. T*he target of miR160a is ARF16*, whose *Arabidopsis* homolog is involved in innate immune responses against bacteria (Li et al. 2014). Campo et al. (2013) reported that when rice leaf tissues are treated with fungal elicitors, certain group of miRNAs are highly expressed. *Nramp6* (rice natural resistance-associated macrophage protein 6) gene is targeted by osamiR7695 leading to compromised expression of the gene. In the similar way, several rice miRNAs that were expressed differentially in response to the rice blast fungus were discovered through deep sequencing. Hence it can be summarized that using the transgenic rice plants that overexpressed miR160a, osa-miR7695 or miR398b could increase the resistance towards the fungal infection (Campo et al. 2013; Li et al. 2014).

On the onset of the viral invasion, some miRNAs were expressed. This was reported by Guo et al. (2012) who identified five upregulated and two downregulated miRNAs in rice samples that were infected by RSV (rice stripe virus). The

abovementioned examples specify that a regulatory system is possessed by rice that integrates the function of miRNA in regulating the immunity in rice against several pathogens.

Recent studies have also shown that mir169 and mir319 are novel factors that ascertain the relationship between rice and *M. oryzae*. Overexpression of mir319 significantly suppresses the immune response in rice by suppressing the jasmonic acid pathway (Zhang et al. 2018). In contrast to the positively regulating miRNAs, miR169 negatively regulates the expression of nuclear factor Y-A (NF-YA) genes acting as downregulator in rice immunity against the rice blast fungus (Li et al. 2017b).

7 Concluding Remarks and Future Prospects

The pathogens causing the plant diseases are a worldwide threat to the rice production. On the other hand, rice has gained a lot of attention to study the disease resistance and other agronomically important traits since its publicly availability of the sequence information. Extensive data have shown that various genomic tools can explore host-pathogen interaction in rice. Our understanding of genomics approaches in relation to the immunity in plants seems to be incomplete. With respect to the other model plants like *Arabidopsis* and tobacco, lesser information has been gathered with reference to disease resistance in crop model rice despite of the various ongoing research activities.

The modern molecular biology and genetic engineering approaches can be very effective in improving the host resistance response. But the emerging virulent races of pathogen can generally break down such responses. The genes associated with the host-defence pathway and the signalling cascades involved in disease resistance can be identified by the latest molecular biology techniques. Therefore, understanding the signalling genes is important for the development of rice cultivars with sustainable and broad-spectrum resistance against various pathogenic microorganisms. From the global climatic aspect, long-term durability and broad-spectrum resistivity in rice are the urgent necessities. This can be accomplished through several emerging approaches like host plant immunity, non-host resistance, interspecific gene transfer, multigene varieties and gene editing.

Many important conclusions have been derived regarding the rice-pathogen interactions via various tools and techniques that include association studies, plant breeding, marker-assisted selection, overexpression studies, loss of function analysis, etc. In relation to the disease resistance, there has been a diverse range of discovery starting from hypersensitive response by *R (resistance) gene*, activation of *PR* gene, hormone biosynthesis of hormone, production of reactive oxygen species (ROS) and their crosstalk with other signal pathways. Even though the genes responsible for the disease resistance in rice are still unclear, further investigation on rice-pathogen interactions on molecular level will provide significant applications to design novel strategies to control the disease, thus improving the rice yield.

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Genetic Engineering and Genome Editing Strategies to Enhance Diseases Resistance of Rice Plants: A Review of Progress and Future Prospects

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Abstract

The occurrence of rice diseases threatens food production worldwide. Developing host resistance is considered as the most efficient and environment-friendly method to reduce yield losses due to the diverse group of pathogens. Disease-resistant quantitative trait loci (QTLs) are a valuable resource for rice crop improvement program. Advanced molecular biology and biotechnological tools accelerated the study of host-pathogen interactions and have resulted in the identification, cloning, and characterization of many genes involved in the plant defense responses. The extent of disease reduction varies with the strategy employed as well as with the characteristics of the pathogen. Manipulation of different hormone levels in transgenic rice plants has provided interesting findings with regard to enhanced disease tolerance or susceptibility. The knowledge is being utilized to modify rice genome to develop disease resistance by means of genetic engineering and CRISPR/Cas9-mediated genome editing technologies. Combinatorial effects of more than one defense genes have been proved to be more promising in conferring disease resistance than single-transgene introduction. The use of tissue-specific or pathogen-inducible promoters and the engineered expression of resistant or susceptibility genes that induce defense responses have the potential to provide commercially useful

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broad-spectrum resistance in the distant future. The issues and challenges of genetic engineering and genome editing to engineer rice disease resistance that need to be addressed are highlighted.

Keywords

Genetic engineering · Genome editing · Disease resistance · CRISPR/Cas9 · Quantitative trait loci (QTL) · *Oryza sativa* · Biotic stress

1 Introduction

Rice is one of the leading primary staple foods for the increasing world population, particularly in Asia. To meet the increasing global food demand, we will have to produce up to 40% more rice by 2030 (Khush 2005). We have to achieve the goal on a reduced sowing space because of urbanization and increasing environmental pollution. Improvement of yield per plant is not the only way to achieve this goal; reduction of losses by biotic and abiotic stress is also a potent solution. According to Food and Agriculture Organization estimates, diseases, insects, and weeds cause the maximum amount of annual yield losses in cereal crops (Khush 2005). In particular, fungal diseases can cause yield losses between 1% and 10%, regionally (Savary et al. 2000). Strong efforts have been invested across the world for improving disease resistance. Most of the efforts are capitalizing on the vast amount of information generated from studying different aspects of plant diseases.

Since the initial definition of the plant resistance (*R*) genes by Flor (1942), several *R* genes are known. The majority of the known *R* genes composed of proteins carrying nucleotide-binding sites and leucine-rich repeat motifs (NBS-LRR) (Jones and Dangl 2006). Most *R* genes recognize pathogen effectors, although there are some exceptions (Lee et al. 2009). Some of these effectors thus correspond to the initial definition by Flor of the avirulence gene. Depending on the presence/absence of the *R* gene and of the matching avirulence product, the interaction will be incompatible or compatible. Many *R* genes have been identified in rice and most code for *NBS-LRR* genes (Ballini et al. 2008). After recognition mediates by the *R* sequence, signal transduction occurs and requires regulators such as MAP kinases (Mishra et al. 2006). Finally, transcription factors like WKRYs modulate a transcriptional reprogramming within the cell (Eulgem 2005), leading to the activation of defense responses. These in term induce the production of secondary metabolites (Peters 2006), pathogenesis-related (PR) proteins (van Loon et al. 2006), strengthening of cell wall (Hückelhoven 2007), and programmed cell death leading to a hypersensitive response (HR) within the cell (Greenberg and Yao 2004).

Resistant cultivars and application of chemical pesticides have been widely used for disease control in practice. However, the useful life span of many resistant cultivars is only a few years, due to the breakdown of the resistance in the face of high variability of the pathogen population. Use of pesticides is costly as well as environmentally undesirable. Thus, novel ways offering protection for an extended time and over a broad geographical area are required. Such strategies will be particularly important in cases where the source of resistance is not available.

The most vital advancement within the space of vertical development for resistance is that the use of the techniques of recombinant DNA technology to develop transgenic plants immune to disease. Moreover, genome editing by programmable sequence-specific nucleases (SSN) like the zinc-finger nucleases (ZFNs) (Bibikova et al. 2003), transcription activator-like effector nucleases (TALENs) (Moscou and Bogdanove 2009), and Cas proteins (Jinek et al. 2012) has the potential to play a significant role in developing disease-resistant plants. Since ZFNs and TALENs are costly and not easy and straightforward to use, these two technologies have not become the method of choice. On the contrary, the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) system simplifies the operation of genome editing and provides a convenient and powerful tool for genome editing. The CRISPR/Cas methods have gained rapid popularity, and it is being used in rice functional genomics and disease resistance breeding (Molla and Yang 2019; Shao et al. 2017; Shen et al. 2017).

2 Genetic Engineering of Rice for Biotic Stress Resistance

Among all the diseases recorded so far, the blast (*Magnaporthe grisea*), bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*), and sheath blight (*Rhizoctonia solani*) are the most serious constraints of rice production. Several methods are established for developing and raising rice resistance against the disease caused but fungus and bacteria through transgenic approaches. In this section, we describe different R genes identified from rice plants and other defense genes utilized for improving rice disease resistance.

2.1 Rice Disease Resistance (*R*) Genes

Biotechnological tools have been playing an instrumental in identifying rice disease resistance genes. Till now, more than 100 major blast resistance (*R*) genes have been identified, and 35 genes have been cloned successfully (Wang et al. 2017). Table 1 summarizes the cloned blast resistance genes. Similarly, for bacterial blight, a total of 42 resistance (R) genes identified and 9 have been molecularly cloned (Vikal and Bhatia 2017). Please see Table 2 for all bacterial blight resistance genes identified. Unlike blast and bacterial blight diseases, no resistance gene has been identified for rice sheath blight (Molla et al. 2019a, b).

2.2 Other Defense Genes from Rice and Non-Rice Sources Utilized for Improving Disease Resistance

Genes from plants apart from rice have been extensively tested in rice. Since no resistant rice germplasm is known and resistance genes have not been identified for sheath blight disease, genes that do not fall in *R* gene category have been utilized for

Table 1 Summary of the cloned blast resistance genes

R gene	Encoding protein	Chromosome	Donor	References
Pi37	NLR	1	St. No. 1	Lin et al. (2007)
Pit	NLR	1	K59	Hayashi and Yoshida (2009)
Pish	NLR	1	Nipponbare	Takahashi et al. (2010)
Pi35	NLR	1	Hokkai 188	Fukuoka et al. (2014)
Pi64	NLR	1	Yangmaogu	Ma et al. (2015)
Pi-b	NLR	2	Tohoku IL9	Wang et al. (1999)
pi21	Proline-rich metal binding protein	4	Owarihatamochi	Fukuoka et al. (2009)
Pi63/ Pikahei-1 (t)	NLR	4	Kahei	Xu et al. (2014)
Pi9	NLR	6	75-1-127	Qu et al. (2006)
Pi2	NLR	6	Jefferson	Zhou et al. (2006)
Piz-t	NLR	6	Zenith	Zhou et al. (2006)
Pi-d2	B lectin receptor kinase	6	Digu	Chen et al. (2006)
Pi-d3	NLR	6	Digu	Shang et al. (2009)
Pi25	NLR	6	Gumei2	Chen et al. (2011)
Pid3-A4	NLR	6	A4 (*Oryza rufipogon*)	Lü et al. (2013)
Pi50	NLR	6	Er-Ba-zhan (EBZ)	Zhu et al. (2012)
Pigm	NLR	6	Gumei4	Deng et al. (2017)
Pi36	NLR	8	Kasalath	Liu et al. (2007)
Pi5	NLR	9	RIL260	Lee et al. (2009)
Pii	NLR	9	Hitomebore	Takagi et al. (2013)
Pi56	NLR	9	Sanhuangzhan No. 2	Liu et al. (2013)
Pi54	NLR	11	Tetep	Sharma et al. (2005, 2010)
Pikm	NLR	11	Tsuyuake	Ashikawa et al. (2008)
Pb1	NLR	11	Modan	Hayashi et al. (2010)
Pik	NLR	11	Kusabue	Zhai et al. (2011)
Pik-p	NLR	11	K60	Yuan et al. (2011)
Pia	NLR	11	Sasanishiki	Okuyama et al. (2011)
Pi1	NLR	11	C101LAC	Hua et al. (2012)
Pi54rh	NLR	11	*Oryza rhizomatis* (nrcpb 002)	Das et al. (2012)
Pi-CO39	NLR	11	CO39	Cesari et al. (2013)
Pi54of	NLR	11	*Oryza officinalis* (nrcpb004)	Devanna et al. (2014)
PiK-h	NLR	11	K3	Zhai et al. (2014)

(continued)

Table 1 (continued)

R gene	Encoding protein	Chromosome	Donor	References
Pike	NLR	11	Xiangzao143	Chen et al. (2015)
Piks	NLR	11	Unknown	GenBank: AET36547.1, AET36548.1
Pi-ta	NLR	12	Yashiro-mochi	Bryan et al. (2000)

enhancing ShB resistance (Molla et al. 2019b). However, more than 50 genes regulating disease resistance have now been discovered from different plant species (Hammond-Kosack and Parker 2003). Some of these genes may not work properly in rice for some biological reasons. Transferring gene from one species to another may lead to detrimental effects. One of the most notable is the central regulatory gene *NPR1* (Cao et al. 1998). Phenotypic cost has been observed when the *Arabidopsis NPR1* gene was transferred to rice (Fitzgerald et al. 2004). The rice plants overexpressing *AtNPR1* displayed an environmentally regulated and heritable lesion mimic phenotype. Moreover, a recent report on *OsWRKY45* demonstrates that overexpression in *japonica* rice confers increased susceptibility to bacterial blight, whereas overexpressing in *indica* rice variety confers increased resistance to bacterial blight. These findings revealed that one should be careful before transferring a gene from one background to another, even within the *Oryza sativa* species.

2.3 Pathogenesis-Related (PR) Proteins

Pathogenesis-related (PR) proteins are a unique category of novel proteins synthesized and accumulated in infected plant tissues. Two well-known PR proteins are hydrolytic enzymes, chitinase, and ß-1,3-glucanase. Hydrolysis of cell wall generates chitin oligomer which is known to induce host defense mechanism. Genes encoding chitinase or ß-1,3-glucanase from plants and microbes have been extensively studied and used in the generation of transgenic rice resistant against fungal pathogens (Punja 2006). Transgenic plants overexpressing either a rice chitinase or a rice thaumatin-like protein showed enhanced resistance against *R. solani* (Datta et al. 1999, 2000, 2001). Green tissue-specific expression of rice *oxalate oxidase 4* (PR-9 family of proteins) gene in transgenic rice showed improved resistance against sheath blight pathogen *Rhizoctonia solani* (Molla et al. 2013). Hydrolytic enzymes from microbial origin have also been demonstrated to be effective in engineering rice disease resistance against fungal pathogens. Bacterial chitinase *ChiC* from *Streptomyces griseus* showed clear inhibition on fungal hyphae under in vitro condition (Itoh et al. 2003). Majority of transgenic rice plants expressing *ChiC* had higher resistance against *M. grisea* than non-transformed control plants (Itoh et al. 2003). Three important genes, namely, *ech42*, *nag70*, and *gluc78* which encode hydrolytic enzymes from *Trichoderma atroviride*, were introduced in rice either singly or in combination. Transgenic plants overexpressing

Table 2 Summary of bacterial blight resistant genes in rice

Xa gene	Resistance to Xoo race	Donor cultivar	Chromosome	References
Xa1	Japanese race-I	Kogyoku, IRBB1	4	Yoshimura et al. (1998)
Xa2	Japanese race-II	IRBB2	4	Sakaguchi (1967)
Xa3/ Xa26	Chinese, Philippine, and Japanese races	WaseAikoku 3, Minghui 63, IRBB3	11	Xiang et al. (2006)
Xa4	Philippine race I	TKM6, IRBB4	11	Yoshimura et al. (1995)
xa5	Philippine race I, II, III	IRBB5	5	Iyer and McCouch (2004)
Xa6	Philippine race 1	Zenith	11	Sidhu et al. (1978)
Xa7	Philippine races	DZ78	6	Sidhu et al. (1978)
xa8	Philippine races	PI231128	7	Vikal et al. (2014)
xa9	Philippine races	Khao Lay Nhay and Sateng	11	Singh et al. (1983)
Xa10	Philippine and Japanese races	Cas 209	11	Mew et al. (1982)
Xa11	Japanese races IB, II, IIIA, V	IR8	3	Ogawa and Yamamoto (1986)
Xa12	Indonesian race V	Kogyoku, Java14	4	Ogawa et al. (1974)
xa13	Philippine race 6	BJ1, IRBB13	8	Chu et al. (2006)
Xa14	Philippine race 5	TN1	4	Taura et al. (1987)
xa15	Japanese races	M41 mutant	–	Nakai et al. (1998)
Xa16	Japanese races	Tetep	–	Noda and Ohuchi (1989)
Xa17	Japanese races	Asominori	–	Ogawa et al. (1989)
Xa18	Burmese races	IR24, Miyang23, Toyonishiki	–	Ogawa and Yamamoto (1986)
xa19	Japanese races	XM5 (mutant of IR24)	–	Taura et al. (1991)
xa20	Japanese races	XM6 (mutant of IR24)	–	Taura et al. (1992)
Xa21	Philippine and Japanese races	*O. longistaminata*, IRBB21	11	Song et al. (1995)
Xa22	Chinese races	Zhachanglong	11	Lin et al. (1996)
Xa23	Indonesian races	*O. rufipogon* (CBB23)	11	Zhang et al. (1998)
xa24(t)	Philippine and Chinese races	DV86	2	Mir and Khush (1990)
xa25/ Xa25(t)/ Xa25	Chinese and Philippine races	Minghui 63, HX-3 (somaclonal mutant of Minghui 63	12	Amante-Bordeos et al. (1992)
xa26(t)	Philippine races	Nep Bha Bong		Lee et al. (2003)

(continued)

Table 2 (continued)

Xa gene	Resistance to Xoo race	Donor cultivar	Chromosome	References
Xa27	Chinese strains and Philippine race 2–6	*O. minuta* IRGC 101141, IRBB27	6	Gu et al. (2004)
xa28(t)	Philippine race 2	Lota sail	–	Lee et al. (2003)
Xa29(t)	Chinese races	*O. officinalis* (B5)	1	Tan et al. (2004)
Xa30(t)	Indonesian races	*O. rufipogon* (Y238)	11	Jin et al. (2007)
xa31(t)	Chinese races	Zhachanglong	4	Wang et al. (2009)
Xa32(t)	Philippine race	*Oryza australiensis* (introgression line C4064)	11	Zheng et al. (2009)
xa33(t), Xa33(t)	Thai races	Ba7 *O. nivara*	6	Korinsak et al. (2009), Natarajkumar et al. (2010)
Xa34 (t) Xa34 (t)	Thai races	Pin Kaset *O. brachyantha*	–	Korinsak et al. (2009), Ram et al. (2010)
Xa35(t)	Xa35 (t) Philippine races	*Oryza minuta* (Acc. No. 101133)	11	Guo et al. (2010)
Xa36(t)	Philippine races	C4059	–	Miao et al. (2010)
Xa38	Indian Punjab races	*O. nivara* IRGC81825	–	Cheema et al. (2008)
Xa39	Chinese and Philippines races	FF329	11	Zhang et al. (2014)
Xa40(t)	Korean BB races	IR65482-7-216-1-2	11	Kim et al. (2015)
xa41(t)	Various Xoo strains	Rice germplasm	–	Hutin et al. (2015)
xa42	Japanese Xoo races	XM14, a mutant of IR24	3	Busungu et al. (2016)

Gluc78 showed enhanced resistance against *M. grisea*, while overexpression of endochitinase gene *ech42* in transgenic rice showed significant resistance against *R. solani*, resulting in 62% resistance against sheath blight disease (Liu et al. 2004). There was a clear co-relation between ech42 expression and chitinase activity with disease resistance (Liu et al. 2004).

2.4 Antimicrobial Proteins

Antimicrobial peptides (AMP) are amphipathic small molecules with conserved α-helix and anti-parallel β-plated sheet and discrete patches of hydrophobic residues

resulting in a structure capable of forming ion channels through the membrane. Majority of antimicrobial peptides contain cysteine residues which are joined to form disulfide bonds, leading to a compact structure. Different types of AMP have been identified from plant as well as microbes and exploited in molecular improvement of rice resistance against fungal and bacterial pathogens. Various types of antimicrobial peptides have been identified in plants, including thionins (Bohlmann and Broekaert 1994), maize zeamatin (Malehorn et al. 1994), coffee circulin (Tam et al. 1999), and wheat puroindoline (Krishnamurthy et al. 2001). Plant defensins are small peptides (45–54 amino acids) that share common characters among plants, insects, and mammals. Dm-AMP1 from *Dahlia merckii*, a defensin, was introduced into rice. Transgenic rice plants expressing Dm-AMP1showed significantly enhanced resistance against *M. oryzae* and *R. solani* but not accompanied by an activation of *PR* gene (Jha et al. 2009). In another study, overexpression of wasabi defensin or *Mirabilis jalapa* antimicrobial protein *Mj-AMP2* gene in transgenic rice exhibited significant resistance against rice blast fungus (Kanzaki et al. 2002). There was 50% reduction in lesions size of the transgenic plants as compared to non-transformed control (Kanzaki et al. 2002). These reports highlight that expression of defensin in transgenic rice has the potential to provide broad-spectrum disease resistance against fungal pathogens. An antifungal protein (AFP) from *Aspergillus giganteus* showed in vitro antifungal activity against diverse economically important fungal pathogens including *M. grisea* (Hagen et al. 2007). The AFP protein from transgenic plants showed inhibitory activity on the in vitro growth of *M. grisea* and therefore enhanced resistance against blast disease (Coca et al. 2004). Transgenic rice plants constitutively expressing AFP protein exhibited inheritance of the transgene in subsequent generation without any phenotypic cost (Coca et al. 2004). Puroindolines, another small protein, reported to have in vitro antimicrobial activity. Transgenic rice plants with constitutively expressing wheat puroindoline genes *PinA* and/or *PinB* were generated. Puroindolines from leaf extracts of the transgenic rice plants reduced the in vitro growth of *M. grisea* and *R. solani*. Transgenic rice expressing *PinA* and/or *PinB* exhibited significantly increased resistance to *M. grisea* and *R. solani* (Krishnamurthy et al. 2001). Cecropins, a family of antimicrobial peptides, constitute a key component of insect immune response. The transgenic rice plants overexpressing cecropin A accumulated active cecropin A protein and showed resistance to rice blast disease (Coca et al. 2006). Similarly, transgenic rice plants overexpressing *cecropin B* gene revealed a significant reduction in lesion development of bacterial blight (Sharma et al. 2000). Oat thionin, when introduced into rice, showed potential to control bacterial leaf blight, caused by *Burkholderia plantarii* (Iwai et al. 2002). Plant defensin genes from *B. oleracea* and *B. campestris* conferred enhanced resistance in transgenic rice to blast and bacterial leaf blight (Kawata et al. 2003). Generally; it has been seen that constitutively expressed antimicrobial proteins in transgenic rice provide partial or moderate but not absolute resistance against disease-causing pathogens.

2.5 Defense Signaling Genes and Broad-Spectrum Disease Resistance

Broad-spectrum resistance is defined at two different levels, i.e., firstly, resistance to different isolates of the same pathogen localized at different regions of the world, and secondly, resistance to two or more unrelated pathogenic strains. Some of the known rice *R* genes have been found to confer broad-spectrum disease resistance against different races of a pathogen and thus have the potential to be used in breeding program or transferred into suitable elite rice varieties through genetic engineering. One of the novel strategies for broad-spectrum plant disease resistance has been to exploit the defense signaling network that modulates the innate plant defense mechanisms against pathogen (Jones and Dangl 2006). Functional genes or proteins belong to both plant and non-plant origins that positively regulate the broad-spectrum systemic acquired resistance against viruses, bacteria, and fungi will act as a useful source for genetic engineering. Recent studies have elucidated that salicylic acid (SA)- and ethylene (ET)/jasmonic acid (JA)-mediated signaling pathways, which act as prime candidate for activation of defense responses against biotrophic and necrotrophic pathogens, respectively, play important roles in rice disease resistance (Glazebrook 2005). Distinct mechanisms might be required for activation of defense responses in rice against different pathogens (Ahn et al. 2005). NPR1 is a master regulator in the SA-mediated signaling pathway in *Arabidopsis thaliana*. Transgenic rice plants expressing *AtNPR1* exhibited enhanced disease resistance against *M. grisea* and *X. oryzae* by modulating the expression of SA-responsive endogenous *PR* genes (Chern et al. 2001; Fitzgerald et al. 2004; Quilis et al. 2008). Tissue-specific expression of *AtNPR1* gene in transgenic rice showed enhanced and significant resistance to the sheath blight pathogen *Rhizoctonia solani* without any detrimental effect on rice phenotype (Molla et al. 2016). *OsNPR1* is a rice orthologue of *Arabidopsis NPR1*. Five NPR1-like genes present in rice genome, and three among them, namely, *OsNPR1*, *OsNPR2*, and *OsNPR3* were induced upon infection by *X. oryzae* pv. *oryzae* and *M. grisea*. Constitutive overexpression of *OsNPR1* in rice conferred disease resistance against bacterial blight but also showed enhanced herbivore susceptibility (Chern et al. 2005). *OsNPR1* might be a potential candidate gene that mediates crosstalk between the SA and JA signaling pathways and provides an approach for engineering rice plants against several diseases (Yuan et al. 2007). Genetic manipulation of JA biosynthesis pathway had shown to improve rice disease resistance against microorganisms. Previous study has shown that transgenic rice plants overexpressing a pathogen-inducible allene oxide synthase (*OsAOS2*) gene, which encodes a key enzyme in the JA biosynthetic pathway, upregulated expression of several *PR* genes and provide significant resistance against *M. Grisea* (Mei et al. 2006). Another study demonstrated that modification of JA-related fatty acid metabolism by suppressing beta-3 fatty acid desaturases, allene oxide cyclase, and 12-oxo-phytodienoic acid reductase exhibited increased disease resistance in transgenic rice against *M. grisea* (Yara et al. 2007, 2008).

2.6 Reactive Oxygen Species

Oxidative burst is a general phenomenon, mediated by hydrogen peroxide (H_2O_2), which has been recognized as a key component of the plant defense after infection. Glucose oxidase (GOX), an enzyme predominantly occurring in some microorganisms, brings about the oxidation of beta-D-glucose, generating H_2O_2, and gluconic acid. Transgenic rice plants transformed with *Aspergillus niger GOX* gene exhibited elevated levels of cellular H_2O_2, which in turn lead to cell death and activation of several defense responsive genes. The overexpression of GOX in transgenic rice plants exhibited enhanced resistance against both *M. grisea* and *X. oryzae* pv. *oryzae* (Kachroo et al. 2003). Similarly, enhanced H_2O_2 generation in infected rice plants with overexpressed *oxalate oxidase* gene showed improved resistance to sheath blight pathogen (Molla et al. 2013).

2.7 Microbe-Derived Elicitor Genes

Microbe-derived elicitor molecules are well-known plant defense activators. Broad-spectrum disease resistance could be achieved by expressing microbial genes coding for elicitors. Several proteinaceous elicitors from microbial origin have been shown to elicit systemic acquired resistance in plants by the activation of SA- and ET/JA-mediated defense signaling pathways. The bacterial harpin and flagellin have been extensively studied for generating broad-spectrum disease resistance in rice through genetic engineering. Recently, a harpin-encoding gene *hrf1*, derived from *X. oryzae* pv. *oryzae*, has been transferred into rice, and the generated transgenic rice lines showed high level of resistance to major races of *M. grisea*. Defense responses including elevated expression of several *PR* genes, increased content of silicon in leaves of overexpressing transgenic plants, and significant inhibition of mycelial growth on leaves of the transgenic rice plants were observed in *hrf1* transgenic plants (Shao et al. 2008). This study revealed that harpins from phytopathogenic bacteria may offer new possibilities for generating broad-spectrum disease resistance in rice. In a similar note, the *flagellin* gene from *Acidovorax avenae*, a phytopathogenic bacterium, was introduced into rice to produce flagellin. The resultant transgenic plants exhibited increased expression of defense genes, elevated H_2O_2 production, and programmed cell death, signifying that the flagellin triggers innate plant immune responses. Flagellin transgenic rice plants exhibited enhanced resistance against *M. grisea*, accounting that the flagellin might provide a novel strategy for developing genetically engineered disease-resistant rice (Takakura et al. 2008).

2.8 Gene Pyramiding in Rice for Biotic Stress Tolerance

The newly released varieties lost their resistance quickly due to the high level of genetic instability in pathogen population. One way to combat this problem is to develop transgenic rice varieties with (i) a combination of genes encoding disease-

resistant proteins which showed synergistic interaction between themselves to realize effective resistance against a particular or group of disease or (ii) pyramiding of genes associated with different diseases for broad-spectrum disease resistance. A previous report showed that pyramiding of three genes, namely, *Xa21*, *chitinase*, and *Bt-fusion* gene in IR72 rice variety through crossing of two independent homozygous transgenic rice lines, provide significant resistance against *X. oryzae* pv. *oryzae*, *R. solani*, and yellow stem borer (Datta et al. 2002). Using both marker-assisted breeding and genetic transformation yielded superior rice lines resistant against blast and leaf blight through pyramiding of *Pi1*, *Piz5*, and *Xa21* (Narayanan et al. 2004). Genetic transformation of rice with a maize ribosome-inactivating protein and a rice *chitinase* gene exhibited enhanced resistance against three fungal pathogens, such as *R. solani*, *Bipolaris oryzae*, and *M. grisea* (Kim et al. 2003). Constitutive co-expression of rice chitinase and thaumatin-like protein in indica rice cultivar resulted in significant enhanced level of resistance against *R. solani* (Kalpana et al. 2006). Similarly, transgenic rice plants pyramided with *chi11*, tlp, and *Xa21* exhibited an enhanced resistance against both sheath blight and bacterial blight diseases (Maruthasalam et al. 2007). Tissue specific co-expression of rice *oxalate oxidase* and *chitinase* genes in transgenic BR-29 rice lines conferred significantly enhanced resistance against *R. solani* (Karmakar et al. 2016). In another report, it has been shown that the dual gene expression cassette harboring *Arabidopsis NPR1* (*AtNPR1*) and rice *chitinase* genes provide a superior level of resistance against sheath blight pathogen *R. solani* than the level of resistance from the individual gene cassette (Karmakar et al. 2017). Combinatorial expression of *chitinase* and *1,3-glucanase* genes in indica rice showed enhanced resistance against sheath blight pathogen, *R. solani* (Sridevi et al. 2008). Transgenic rice lines expressing four antifungal genes, i.e., *RCH10*, *RAC22*, *Glu*, and *B-RIP* showed a heightened state of resistance to *M. grisea*, rice false smut (*Ustilaginoidea virens*), and rice kernel smut disease (*Tilletia barclayana*) (Zhu et al. 2007). Therefore, an ingeniously planned genetic engineering strategy involving a balanced expression of different transgenes with a potential different mode of action would ensure broad-spectrum and durable tolerance against diverse group of pathogens.

3 Genome Editing System

Genome editing systems with engineered nuclease (GEEN) allow cleavage and rejoining of DNA molecules in specified target sites to successfully modify the genetic loci. Special enzymes such as restriction endonucleases (RE) and ligase can be used for cleaving and rejoining of DNA molecules in small genomes like bacterial and virus. However, using only these two enzymes such as restriction endonucleases and ligases, it is very difficult to manipulate large and complex genomes of higher organisms, including plants. Target specificity of RE is enough for short DNA sequences such as bacterial and viral genomes, it is not sufficient to work with large genomes such as plant.

Invention of engineered nucleases for genome editing revolutionized biological study. There are three well-known nucleases such as zinc finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN), and CRISPR/Cas9 available as genome editing tools. ZFN and TALEN depend on protein-DNA interaction, whereas CRISPR/Cas9 relies on RNA-DNA interaction through Watson-Crick base pairing. These engineered nucleases bind to targeted loci of the genome and make a highly specific double-strand break (DSB). Upon recognition of the DSB, the error-prone cellular repair machinery inserts or deletes few nucleotides at the DSB. Due to this indel (insertion/deletion) formation, the targeted gene suffers from frameshift mutation and that ultimately causes knockout of the gene. Similarly, utilizing cellular homology-directed repair (HDR) system, precise editing could be achieved with additional supply of donor template with homologous arms. Since working with CRISPR/Cas9 is the simplest among the three tools, it gains rapid popularity within a very short period of time. All the abovementioned three tools are discussed below briefly.

3.1 Tools Available for Editing Rice Genome

3.1.1 Zinc Finger Nucleases (ZFNs)

ZFNs (zinc finger nucleases) are the first-generation genome editing tools, which are chimerically engineered nucleases, and developed after the discovery of the working principles based on functional Cys_2-His_2 zinc finger (ZF) domain (Kim et al. 1996). Each Cys_2-His_2 ZF domain consists of about 30 amino acid residues, which are capable of binding to target DNA by inserting a α-helix of the protein into the major groove of the DNA-double helix (Pavletich and Pabo 1991). Each zinc finger (ZF) protein has the ability to recognize three tandem nucleotides in the target DNA. ZFN monomer consists of about two different functional domains: an artificial zinc finger (ZF) Cys_2-His_2 domain at the N-terminal portion and a FokI DNA cleavage domain at the C-terminal region (Fig. 1). Dimerization of FokI domain is critical factor for ZFN enzymatic activity (Kim et al. 1996). The modular recognition of zinc finger domains represents consecutive three bp targets enabled the realization that each of the individual zinc finger domains could be interchangeable and manipulation of the domains would lead to unique binding specificities to the proteins, enabling targeting of specific unique sequences in the genome.

The application of ZFNs involves assembly, optimization, and modular design of zinc fingers against specific target DNA sequences. Over the past few years, zinc finger domains have been generated to recognize a large number of triplet nucleotides, which provide the accurate selection and linking of zinc fingers with a particular sequence that would permit recognition of the target sequence. Many successful studies on genome editing in plants have been reported using zinc finger nucleases (ZFNs). Utilization of ZFNs to induce a double-strand break in the soluble *starch synthase* gene (*SSIVa*) in rice leads to the regulation of the *SSIVa* expression. ZFN-mediated targeted gene disruption in the coding sequence of the *SSIVa* rice gene is an effort to elucidate the functional role of the gene (Jung et al. 2018).

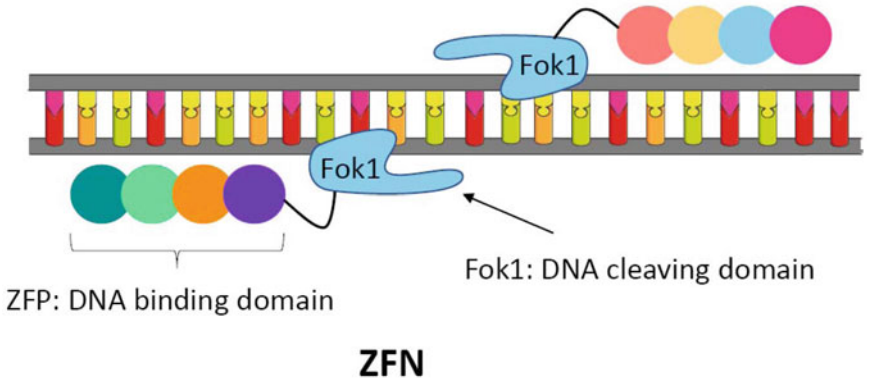

Fig. 1 Basic structure and design of a zinc finger nuclease (ZFN). ZFNs are created by joining a DNA-binding region to the catalytic domain of the nonspecific Fok1 endonuclease. Each zinc finger, illustrated by an individual circle, recognizes 3–4 nucleotides, and, by assembling three or four suitable zinc finger motifs, a sequence-specific DNA-binding domain can be created. Fok1 nuclease activity requires dimerization, and so the customized ZFNs function in pairs. As shown, the zinc finger-binding domain brings two Fok1 units together in the right orientation over the target sequence; this induces Fok1 dimerization and target sequence cleavage

3.1.2 Transcription Activator-Like Effector Nucleases (TALENs)

The efficient manipulation of target genomic DNA led to the identification of unique transcription activator-like effector (TALE) proteins that recognize and activate specific plant promoters through a set of tandem repeats which form basis for the creation of a new genome editing tool consisting of chimeric nucleases, called TALE nucleases (TALENs) (Jankele and Svoboda 2014). DNA-binding ability of these proteins was first discovered in the year 2007; after a year later, two scientific groups have decoded the recognition code of target DNA sequence by TALE proteins (Boch et al. 2009).

TALE monomers consist of a central repeat domain (CRD) that provides DNA binding and host specificity. The central repeat domain (CRD) consists of 34 amino acid tandem repeats. Two of the amino acids at positions 12 and 13 of the repeat are highly variable and are responsible for the recognition of specific nucleotide (Fig. 2). These two positions are known as repeat variable diresidue (RVD) (Moscou and Bogdanove 2009). The DNA binding specificity of RVD domain has been repurposed for designing specific DNA binding artificial TALE proteins. The fusion of Fok1 nuclease domain with TALE DNA binding domain has been demonstrated to successfully create a new class of target-specific nucleases (Christian et al. 2010).

With the use of TALENs, it will be possible to introduce double-strand breaks in any location of the genome as long as that location harbors the recognition sequence corresponding to the DNA-binding domains of TALENs.

The pathogen *Xanthomonas oryzae* pv. *oryzae* (Xoo) produces and translocates its virulence proteins with the TAL effectors into the host cells through a type-III secretion system. After internalization, TAL effectors are localized into the nuclei of the host cells and bind to the promoters of susceptibility (*S*) genes. After that, TAL

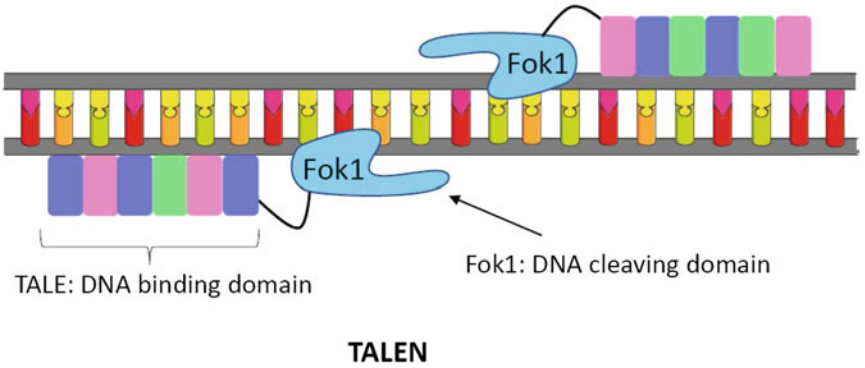

Fig. 2 A scheme for introducing a double-strand breaks using chimeric TALEN proteins. One monomer of the DNA-binding protein domain recognizes one nucleotide of a target DNA sequence. Two amino acid residues in the monomer are responsible for binding. Recognition sites are located on the opposite DNA strands at a distance sufficient for dimerization of the FokI catalytic domains. Dimerized FokI introduces a double-strand break into DNA

effectors activate the S-gene expression that in turn leads to more susceptibility of host plants to bacterial infection. *SWEET11*, *SWEET13*, and *SWEET14* are known rice susceptibility genes (Yang et al. 2006). *SWEET14* gene has been disrupted using TALEN to develop bacterial blight resistant rice plants (Li et al. 2012). Similarly, Cai et al. showed that TALEN-mediated editing of rice gene *Os09g29100* enhances resistance to the bacterial leaf streak pathogen *Xanthomonas oryzae* pv. *oryzicola* (Cai et al. 2017).

3.1.3 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

A novel genome editing system that has been discovered recently and became so demanding and popular is the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein system, popularly known as CRISPR/Cas system. The technology is derived from CRISPR/Cas type II immune system found in the bacterium *Streptococcus pyogenes*. It is comprised of CRISPR RNA (crRNA), trans-activating crRNA (tracrRNA), and Cas9 protein. crRNA-tracrRNA hybrid guides the Cas9 nuclease to bind to a homologous nucleic acid and make a specific double-strand break. Jinek et al. (2012) first demonstrated successfully this system to make targeted DSB in DNA. The study also showed that a single chimeric RNA (comprised of crRNA and tracrRNA) known as single guide RNA (sgRNA) could direct the Cas9 to any DNA sequences of interest if they have a NGG sequence nearby. This 5′-NGG-3′ is known as protospacer adjacent motif (PAM). The 5′ 20 bp sequence in the sgRNA sequence is known as protospacer sequence which can be designed as per the requirement of a specific experiment. Hence, the design of a CRISPR/Cas experiment is easy and straightforward.

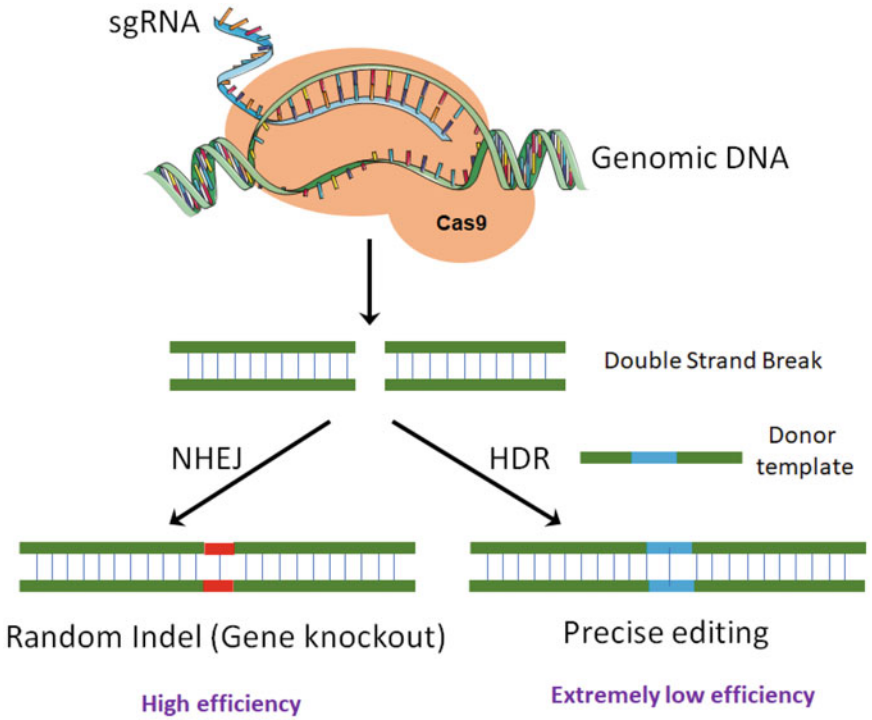

Fig. 3 Schematic depiction of CRISPR/Cas9 genome editing mechanism. sgRNA guides Cas9 to bind and cut specific genomic locus. Once a double-strand break (induced by Cas9) is detected, cellular repair machinery repairs it through either non-homologous end joining (NHEJ) or homology directed repair (HDR) pathways. Error-prone NHEJ causes indel (red) formation at the DSB and results in frameshift of the coding sequence knocking out the gene activity. Although extremely low in efficiency, HDR uses homologous sequence to precisely repair the DSB. If artificial homologous sequence (donor) (green) containing desired nucleotide alteration (blue) is supplied in the vicinity of DSB, HDR could incorporate the change (blue) in the targeted genomic locus

Since the initial study by Jinek et al. (2012), CRISPR/Cas9 system has extensively been used in various fields of applied biology, biotechnology, and genome engineering, due to its simplicity, efficiency, and wide applicability. Besides the conventional CRISPR/Cas9-mediated knockout techniques (Fig. 3), various CRISPR-derived technologies have been generated. CRISPR interference (CRISPRi) and CRISPR activator (CRISPRa) have been generated for gene repression and activation, respectively (Qi et al. 2013; Gilbert et al. 2013). Recently, CRISPR/Cas-mediated base editing systems have been developed to install precise point mutation in the genome (reviewed by Molla and Yang 2019). Base editing system has been used successfully to precisely install A to G conversion in the rice genome (Molla et al. 2020).

Table 3 Use of CRISPR/Cas technology for developing disease-resistant rice

Species	Pathogen	Target gene	Transformation methods	References
Oryza sativa L. japonica	Tungro virus	eIF4G	*Agrobacterium*-mediated transformation	Macovei et al. (2018)
Oryza sativa L. *japonica*	*Magnaporthe oryzae*	SEC3A	Protoplast transformation	Ma et al. (2018)
Oryza sativa L. *japonica*	*Magnaporthe oryzae*	ERF922	*Agrobacterium*-mediated transformation	Wang et al. (2016)
Oryza sativa L. *japonica*	*Xanthomonas oryzae* pv. *oryzae*	SWEET13	*Agrobacterium*-mediated transformation	Zhou et al. (2015)
Oryza sativa L. *japonica* and *Oryza sativa* L. *indica*	*Xanthomonas oryzae* pv. *oryzae*	SWEET11, SWEET13 and SWEET14	*Agrobacterium*-mediated transformation	Oliva et al. (2019)

3.2 CRISPR/Cas9 System for Biotic Stress Tolerance in Rice

CRISPR/Cas9 system has been utilized to install mutation in *OsSWEET13* gene to prevent its neutralization by the TAL effector gene *pthXo2*, leading to improved tolerance against bacterial blight disease (Zhou et al. 2015). A recent study has been demonstrated that CRISPR/Cas9-targeted knockout of an ERF transcription factor gene *OsERF922* showed enhanced resistance against rice blast fungus (Wang et al. 2016). Targeted mutagenesis with insertion or deletion at the target site and the frequency of mutation was up to 42% in T_0 plant lines. Phenotypic assessment of six T_2 homozygous mutant lines demonstrated that there was a significant reduction in the number of blast lesions in mutant lines as compared to wild-type plants. A recent study demonstrated editing of promoters of multiple SWEET genes in rice to develop broad spectrum bacterial blight resistance (Oliva et al. 2019). This result revealed that CRISPR/Cas9 is a powerful tool for enhancing blast resistance in rice. A brief summary of studies on CRISPR/Cas-mediated attempts to develop disease-resistant rice plants is given in Table 3.

4 Future Prospects

In the cases where defense manipulation is achieved by expression of a single or multiple protein from microbial origin or phytoalexins, the resistance in transgenic rice is not absolute, and majority of them only show partial or moderate resistance against a particular disease. Surprisingly, a number of disease resistance genes have been isolated from rice, and few have been shown to provide broad-spectrum disease resistance against diverse groups of pathogens.

Engineering of rice varieties with durable and broad-spectrum resistance would be only achieved probably through genetic manipulation of regulatory mechanisms and signaling network controlling activation of multiple defense-responsive genes. Extensive and through studies of rice disease resistance, using approaches such as genomics and proteomics, will lead to identification of novel candidate genes that are involved in the defense signaling as well as subsequent metabolic pathways. Functional genomics aided by new genome editing technologies would play a significant role toward that direction. These identified novel genes will be helpful in the generation of new superior rice varieties with high level of durable resistance against broad range of disease caused by diverse pathogens.

Knowledge of molecular mechanisms of host-pathogen interaction is crucial to utilize the full potential of the advance technologies like genome editing. Versatile technologies like CRISPR/Cas would assist us to decipher the mechanism in one hand and could be utilized to develop disease-resistant plants utilizing that knowledge on the other hand. Most simplified way is to knock out or knock down any known negative regulator or susceptibility genes for a disease. However, it needs to keep in mind that many susceptibility genes play pleiotropic roles and knocking out may have some unknown consequences. The RVD of bacterial TAL proteins has specific binding sequences in the promoter of susceptibility genes to increase their expression. Instead of knocking out by conventional CRISPR, the nucleotide/s of the TALE binding site in the susceptibility gene promoters can be mutated utilizing CRISPR/Cas base editing technologies to enhance resistance without pleiotropic effects (Molla and Yang 2019). Base editing permits C to T and A to G transitions mutations in plants. This editing tool has tremendous potential in installing precise mutation in the genome. However, changing a susceptible allele to a resistant allele through genome editing may need to perform transversion mutation, specific addition, deletion, or replacement of sequences. Homology directed repair (HDR) (Fig. 3) is the only available way to achieve those kinds of changes in the genome. The matter of concern is that HDR is extremely low in efficiency in plants. However, a recently developed technology, prime editing, can perform all kinds of precise editing up to 40 bp with much higher efficiency than HDR (Anzalone et al. 2019). Rapid advancements in technologies would ease genome modification and subsequently aid in developing disease-resistant rice plants.

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Transgenic Rice Live Against Bacterial Blight

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Abstract

Rice is one of the most essential staple foods for most of the countries in the world. Yield of this important crop is severely hampered worldwide by an increasing number of microbial attacks. Among these, bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* is one of the main constraints of rice production. Different management strategies are promoted to alleviate this serious problem. Conventional breeding practices are mostly utilized to develop resistant cultivars in the different parts of the world as the approaches are cost effective and environment friendly. However, the fruitful results may not be achieved due to low yield and reduced effectiveness against that pathogen. On the other hand, pathogen races are gradually changing their organization to adopt the unfavorable environment. In this present situation, the research efforts have been shifted to find out resistance genes in plants or in others, against specific pathogens. Till date around 40 genes have been detected in rice. Only few genes have been cloned successfully and tested against this devastating pathogen. However, scientists are now concerning about the durability and long-term protection by developing new molecular tools which might also help in sustainable agriculture. In this chapter, we are trying to summarize different aspects of this disease and recent researches on rice plant against this pathogen.

Keywords

Conventional breeding · Sustainable agriculture · Transgenic approaches

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1 Introduction

Rice is one of the most accepted staple crops of almost two thirds of the world and specially for developing countries like India. However, cultivation of this popular crop has been constrained due to rigorous climate change. Rice farming is still challenging due to global population expansion, extensive urbanization, and constant shifting of cultivable land to industrial park. Greater modernization promotes greater abiotic stresses to the plants like rise in temperature, prolonged drought condition, and water stress, increases harmful radiations, upholds reactive oxygen species (ROS) generation, etc. Employment of excessive amount of fertilizers ultimately reduces soil fertility. Use of harmful chemical pesticides and fungicides contaminates groundwater and also encourages pathogens to improve themselves and to develop new races. Application of non-judicious farming technology and mistreatment of high throughput up to date agricultural technologies has ultimately gifted non-cultivable lands (Delteil et al. 2010). To fulfill the rising demand of food supply throughout the world, growers have to produce more than 40% extra rice by 2030 (Khush 2005; Delteil et al. 2010). However, it will be a herculean task today due to tremendous challenging factors. In spite of other abiotic factors, bacterial and fungal pathogens cause a serious threat on rice cultivation (Delteil et al. 2010). Near about 20–30% of rice production is regularly hampered per year globally by various rice pathogens (Chattopadhyay et al. 2017). In this connection, *Xanthomonas oryzae* pv. *oryzae*, a Gram-negative bacterium and the causal organism of bacterial leaf blight of rice, causes almost more than 50% crop loss in respect to others (Ishiyama 1922; Chattopadhyay et al. 2017). Though it is mainly a seed-borne disease, all the developmental stages are susceptible to the pathogen under encouraging environmental situations (Chattopadhyay et al. 2017).

This disease outbreak occurs throughout the world and causes serious crop loss and economic suppression mainly in different parts of Western Africa and Asia. Moreover, intense wind associated with rains may amplify the epidemic of bacterial blight (Chattopadhyay et al. 2017). It basically occurs in high-yielding rice varieties grown in the monsoon season, under profound nitrogen fertilization and especially in the irrigated and rain-feed ecosystems (Laha et al. 2017). Bacterial blight epidemics were reported from different parts of India (Laha et al. 2009, 2017; Yugander et al. 2014). More than 50% crop loss was reported in different West African countries due to this major destructive disease (Basso et al. 2011; Laha et al. 2017). Singh et al. (2013) recorded the highest economic loss in the following series Pusa Basmati-1 (45%) > Haryana Shankar Dhan-1 (31%) > HKR 47 (23%) in India. Besides that, severe economic loss was also noticed in various regions of Southern China, Japan, the Philippines, Pakistan, Nepal, and South and Central American countries (Adhikari and Mew 1991; Mew et al. 1993; Khan et al. 2000; Akhtar et al. 2003; Qi 2009; Corral et al. 2013).

It was the most challenging task to control this devastating pathogen over time. The most convenient, economic, useful, and sustainable method was to generate resistant cultivars against this pathogen (Chattopadhyay et al. 2017). However, selection of wild resistant varieties was tedious, and new varieties of pathogen

races were also challenging over time. Researchers sometimes utilize some abiotic and biotic inducers also, which are able to boost up the innate immunity of the plants. However, it was not so effective in this pathosystem. Nowadays, more researches move on to developing transgenic rice varieties against this pathogen. More than 40 genes have been tested till date to generate elite variety of rice (Chattopadhyay et al. 2017; Laha et al. 2017). By virtue of the advancement of molecular biology and biotechnological methods, multi-genes are incorporated within a single rice variety through gene pyramiding and marker-assisted selection techniques (Chattopadhyay et al. 2017). Consequently, whole genome editing tools like clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas9) and transcription activator-like effector nucleus (TALEN) are also utilized (Chattopadhyay et al. 2017; Laha et al. 2017).

This chapter emphasizes on the detailed outlook of the important disease and also provides information about the various strategies including molecular tools for the improvement of bacterial blight resistance of rice.

2 Geographical Distribution and Past History of Bacterial Blight

The devastating pathogen of bacterial blight disease of rice has been first identified and reported in the year of 1884 from Japan (Tagami and Mizukami 2008; Chattopadhyay et al. 2017). Later on, it was characterized properly and named as *Xanthomonas oryzae* pv. *oryzae* (Ishiyama) Swings et al. (*Xoo*) in the year 1922. Though almost all the rice-growing regions of the Caribbean island like Salvador, Mexico, Costa Rica, Honduras, and Panama were affected by this disease, it was first reported from the Mali region of West Africa (Buddenhagen et al. 1979). In South America, the affected regions include Colombia, Ecuador, Bolivia, and Venezuela (Lozano 1977; Sere et al. 2013). Louisiana and Texas from North America were most vulnerable for this pathogen (Jones et al. 1989). Furthermore, it was accounted from Australia and all the Asian countries (Ou 1985; Devadath 1992; Win et al. 2013). Srinivasan et al. (1959) reported the incidence of this disease from the state Maharashtra in India. However, its occurrence has not been established in Europe. The worldwide distribution of this disease is presented in Fig. 1.

3 Symptoms

It is a representative of typical vascular disease among others and has three discrete phases of symptoms. In the first phase of the disease, it shows pale-green to straw-colored striped water-soaked lesions with wavy margins on the tip of the isobilateral leaves which migrates longitudinally downward (Fig. 2). The leaf blight lesions may appear on both edges of the leaf and ultimately cover the whole leaf in the severe condition. Finally, it turns to whitish to grayish black due to the progression of saprophytic fungal growth. A small opaque yellowish drop containing bacterial

Fig. 1 World distribution of blast disease of rice (Laha et al. 2017)

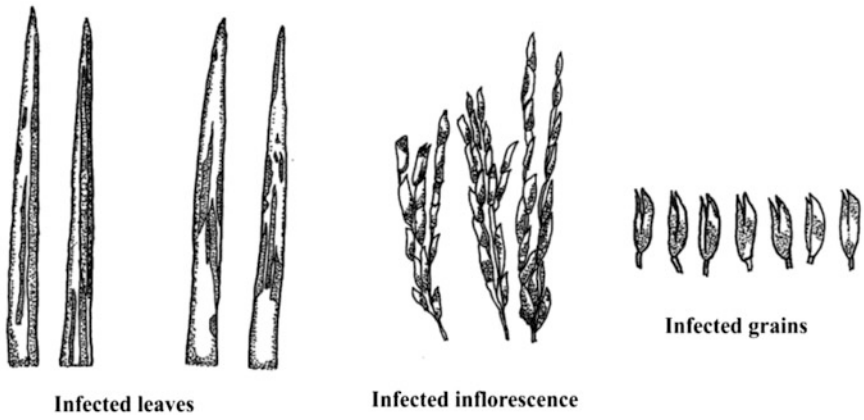

Fig. 2 Symptoms of bacterial blight disease

eluents may be observed in the humid areas at the morning, which dries up and turns into small spherical yellowish beads (Chattopadhyay et al. 2017).

In the tropical countries, kresek or wilt phase is the harshest phase of this disease. In this situation, the leaves become yellow to grayish in color and roll completely with wilting symptoms. In the acute phases, the tillers shrivel, and the affected plants ultimately die (Laha et al. 2017).

On the other hand, in the Philippines, the symptoms appear in the cluster of leaves which turns into pale yellow or whitish, and finally the affected leaves wither and dry up (Laha et al. 2017).

4 Pathogen

Xanthomonas oryzae pv. *oryzae* (Ishiyama) Swings et al. is a rod-shaped, non-spore-forming, Gram-negative, and motile uniflagellate bacterium having a single polar flagellum. It belongs to Xanthomonadaceae (family), Xanthomonadales (order), Gammaproteobacteria (class), and Proteobacteria (phylum) in the domain Bacteria. It secretes xanthomonadin (a brominated aryl polyene pigment), a typical non-diffusible yellow pigment (Laha et al. 2017).

5 Disease Cycle and Epidemiology

According to the inception of different studies on this disease carried out by different scientific organizations like the International Rice Research Institute (IRRI), Manila, Philippines, All India Coordinated Rice Improvement Project (AICRIP), the main cause of the disease is seed infection (Laha et al. 2009). However, there are few such references which indicate that the disease may not occur due to planting of those seeds. Furthermore, it was observed that the infection may occur in the self-grown plants, from the pre-infected stubble straws and also from the infected wild rice varieties. The pathogen may also survive on some wild grasses like *Panicum repens*, *Leersia hexandra*, and *Cyperus rotundus* and contaminate irrigation water which may also act as a source of primary inoculum. Cloudy humid conditions along with moderate temperature like 28–30 °C and excess use of nitrogenous fertilizer aggravate rapid growth and spreading of the disease (Ezuka and Kaku 2000) (Fig. 3).

6 Brief Overview of Disease Management Strategies Employed till Date

Various control measures including host nutrition and physical, cultural, and chemical control are employed to restrict this devastating disease. However, the best possible outcome may not be achieved due to regular modification of the pathogenic races, though improvement of plant resistance by the introduction of new gene or by regular breeding technique may be the best solution to overcome this disease.

Use of cultural control as an essential part of integrated disease management may be useful to some extent to control this disease. Removing infected plant trashes, wild rice plants, and weeds like *Leersia* sp. and *Cyperus* sp. may be quite useful to confine pathogen progress. Restriction of unnecessary pruning of plant parts and field to field irrigation may be helpful to control this disease. Not only that, use of pathogen-free certified seeds and judicious use of manures mainly nitrogen may be beneficial (Ezuka and Kaku 2000; Laha et al. 2009, 2017).

After disease inception, the use of different chemicals may act as SOS to control this destructive disease, though chemicals which are utilized in the field may not be eco-friendly. Till date various chemicals like chloramphenicol, cellomate, Sankel, streptomycin, phenazine, etc. have been used in the field (Laha et al. 2017).

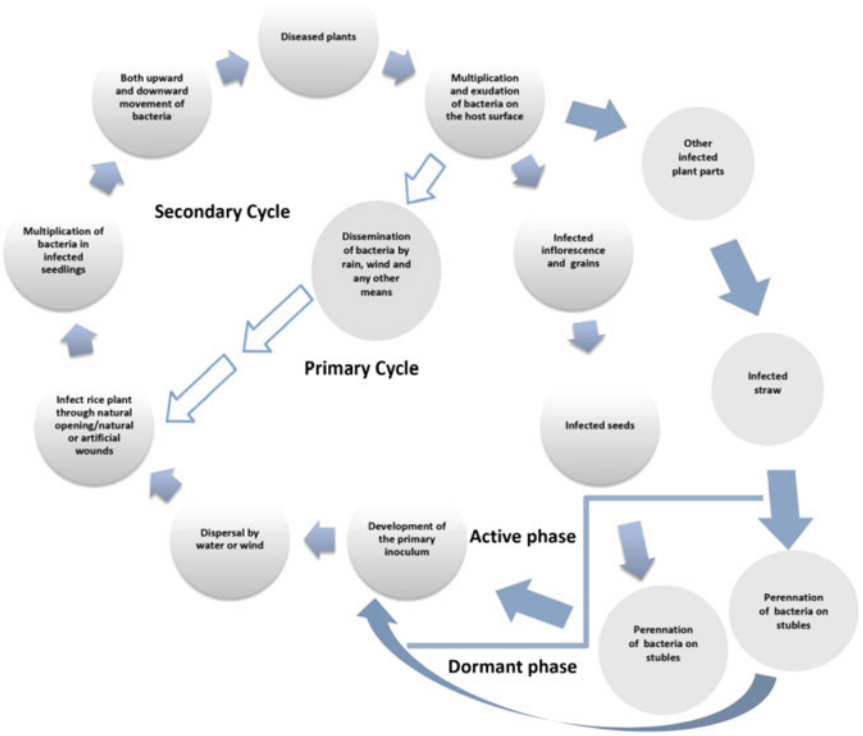

Fig. 3 Disease cycle of bacterial blight (causal organism: *Xanthomonas oryzae* pv. *oryzae*)

However, satisfactory results may not be achieved and sometimes more than one chemical was required to control this pathogen and the remnants of which may cause serious health hazards (Srivastava 1972; Laha et al. 2017). Seed soaking with various chemicals like Agrimycin 100, streptomycin, Terramycin, chlortetracycline hydrochloride, Ceresan, etc. at variable proportions along with hot water treatment may diminish the pathogen inoculum from seeds (Laha et al. 2017). Srivastava (1972) reported that TF-130 and ATDA (2-amino-1,3,4-thiadiazole) were most efficient against this pathogen in Japan and adjoining countries. Agrimycin 100 and Fytolan (copper oxychloride) in a specific proportion (50:500) may be useful to check secondary infection (Singh et al. 1980). According to Laha et al. (2009), 12.5 kg/ha Klorocin application 10 days after transplanting may give useful results. The highest disease control by the application of chemicals was achieved by applying Streptocycline (200 mg/l), copper oxychloride (2.5 g/l), and 2,4-D ethyl ester (1.0 ml/l) in combination (Singh et al. 2012b; Laha et al. 2017).

Crop improvement by conventional breeding methods may also help to combat against this disease. In the year 1969, two bacterial blight rice varieties named IR 20 and IR 22 were developed from IRRI, mostly on the basis of the major resistance genes like *Xa4* (Khush et al. 1989; Laha et al. 2017). However, those high-yielding

varieties (HYVs) showed different degrees of susceptibility in different countries against this pathogen, which may be due to the development of more virulent new pathogenic races (Laha et al. 2017). Consequently, new resistance genes like *Xa5* and *Xa7* have been incorporated by plant researchers (Khush et al. 1989; Laha et al. 2017). Gradually other bacterial blight resistance-related new genes were discovered from wild rice varieties like *Xa21*, obtained from *Oryza longistaminata* (Khush et al. 1990; Laha et al. 2017). Till date more than 35 genes including *Xa23*, *Xa27*, *Xa30(t)/Xa38*, and *Xa33* have been identified from various sources and customized for marker-assisted selection breeding (Sundaram et al. 2014; Kim et al. 2015; Laha et al. 2017). Nowadays, to achieve the highest durable resistance against this destructive pathogen, amalgamation of different genes into a single rice cultivar was taking place by using the process of marker-assisted selection breeding (Laha et al. 2017). By using this technique, various rice varieties were obtained which are listed in Table 1.

Researches on host resistance against this pathogen are not so wide in Africa as in Asia. According to available reports, NERICA 4, NERICA 8, and NERICA 14 varieties showed modest level of bacterial blight resistance (Banito et al. 2012). However, few accessions of *O. glaberrima* from Mali showed high degree of resistance against this pathogen race A3 (Djedatin et al. 2011; Laha et al. 2017).

7 Why Modify the Employment of Bacterial Blight Resistance Genes?

It is well-known that the effective genes present in the elite clones of different rice varieties are evenly distributed all across the vast geographic areas. Sometimes they interact with the existing pathogen community of local inhabitants. Very often, long-term contact with efficient pathogen may cause an epidemic outbreak, which is mainly due to the loss of durability of the resistance genes (Dossa et al. 2015). A detailed understanding of pathogen physiology, host metabolism, and disease epidemiology and consequently knowledge of interdisciplinary approaches are very much necessary to control the pathogen dynamics. Studies on suppressive environments and effective genes through effector biological programs may employ a new arena in the field of integrated disease management. The outcome of those experiments should be properly informed to the forerunner of the cultivation.

Near about 40 genes were identified for bacterial blight of rice mostly from the wild and cultivable varieties (Khan et al. 2014; Zhang et al. 2014; Dossa et al. 2015). In Asia, the resistance genes like *Xa 4*, *Xa 5*, *Xa 13*, and *Xa 21* are mostly used for breeding purposes (Khan et al. 2014). Interestingly, the bacterial blight pathogen uses transcription activator-like (TAL) effectors to colonize the host system. In the large deployment of disease resistance against this pathogen and in contrast to the other pathosystems, *Xa* genes can be further classified into sub-categories (Boch et al. 2014; Dossa et al. 2015). The roles of different *Xa* genes are discussed in Table 2.

Table 1 Different commercialized rice varieties obtained by breeding

S. no.	Name of the rice varieties	Genes involved	Country	References
1.	Samba Mahsuri	*Xa21*, *Xa13*, and *Xa5*	India	Laha et al. (2009)
2.	PR106	*Xa5*, *Xa13*, and *Xa21*	India	Singh et al. (2001)
3.	Type 3 Basmati	*Xa21*, *Xa13*, *sd-1*	India	Rajpurohit et al. (2011)
4.	Lalat and Tapaswini	*Xa21*, *Xa13*, *Xa5*, and *Xa4*	India	Sundaram et al. (2014)
5.	PAU 201	*Xa38*, *Xa13*, *Xa21*	India	Sundaram et al. (2014)
6.	*O. rufipogon*	*Xa39(t)*	India	Sundaram et al. (2014)
7.	Mahsuri	*Xa4*, *Xa5*, *Xa13*, and *Xa21*	India	Guvvala et al. (2013)
8.	Swarna and IR64	*Xa21*, *Xa13*, and *Xa5*	India	Sundaram et al. (2014)
9.	Pusa Basmati-1	*Xa21* and *Xa13*	India	Singh et al. (2012a), Sundaram et al. (2014)
10.	Angke	*Xa4* and *Xa5*	Indonesia	Laha et al. (2017)
11.	Konde	*Xa4* and *Xa7*	Indonesia	Laha et al. (2017)
12.	NSIC Rc142 (Tubigan 7)	*Xa4* and *Xa21*	Philippines	Laha et al. (2017)
13.	NSIC Rc154 (Tubigan 11)	*Xa4* and *Xa21*	Philippines	Laha et al. (2017)
14.	RD6	*Xa5/Blast R*	Thailand	Pinta et al. (2013)
15.	Zhonghui 8006, Zhonghui 218, Guodao 1, Guodao 3, Guodao 6, and II You 8006	*Xa21*	China	Verdier et al. (2012), Rao et al. (2014), Laha et al. (2017)
16.	Minghui 63 (a restorer line)	*Xa21*	China	Chen et al. (2000)
17.	Minghui 63	*Xa7* and *Xa21*	China	Zhang et al. (2006)

Table 2 List of R genes of various rice cultivars securing resistance toward *Xanthomonas oryzae* pv. *oryzae*

Source material	Gene	Ch No.	Nature of resistance	References
Oryza sativa sp. *japonica* (cv. Kogyoku)	*Xa1*	4L	Resistant to Japanese race 1 (race specific dominant)	Yoshimura et al. (1998), Chattopadhyay et al. (2017)
Oryza sativa sp. *indica* (cv. Tetep)	*Xa2*	4L	Resistance to Japanese race 2 (race specific dominant)	He et al. (2006), Chattopadhyay et al. (2017)
Oryza sativa sp. *japonica* (cv. Wase Aikoku 3)	*Xa3/ Xa6/ Xa26*	11	Highly resistant to Philippine races 1, 2, 3, 4, 5, and 9	Xiang et al. (2006), Hur et al. (2013), Chattopadhyay et al. (2017)
Oryza sativa var. *indica* (IR20, IR22, IR64)	*Xa4*	11	Highly resistant to Philippine races 1, 4, 5, 7, 8, and 10 (effective under low temperature)	Yoshimura et al. (1995), Chattopadhyay et al. (2017)
Oryza sativa ssp. *indica* (DV85, DV86, DV78)	*Xa5*	5S	Resistant to Philippine races 1 and 4; but susceptible to race 6 (recessive in nature)	Blair et al. (2003), Iyer and McCouch (2004), Chattopadhyay et al. (2017)
Oryza sativa ssp. *indica* (DZ78)	*Xa7*	6	Dominant but not race specific and effective under high temperature	Porter et al. (2003), Chattopadhyay et al. (2017)
PI231129 (American cultivar)	*Xa8*	7	Recessive provide race-specific resistance to Philippine and North Indian *Xanthomonas oryzae* pv. *oryzae* races at seedling and adult plant phase	Vikal et al. (2014), Chattopadhyay et al. (2017)
Cas 209	*Xa10*	11L	Dominant at all developmental stages; race-specific resistance to Philippine races like PXO86 (R2), PXO112 (R5), and PXO145 (R7)	Yoshimura et al. (1983), Gu et al. (2008), Chattopadhyay et al. (2017)
IR8	*Xa11*	3L	Dominant and provide race-specific resistance to different Japanese races like IB, II, IIIA, and V	Goto et al. (2009), Chattopadhyay et al. (2017)
Oryza sativa sp. *japonica* (cv. Kogyoku)	*Xa12*	4	Dominant and provide race-specific resistance to Philippine race 5 and Japanese race V	Taura et al. (1992b), Chattopadhyay et al. (2017)
Oryza sativa sp. *indica Aus-Boro* line (cv. BJ1)	*Xa13*	8L	Provide Philippine race 6-specific resistance	Sanchez et al. (1999), Chu et al. (2006), Chattopadhyay et al. (2017)

(continued)

Table 2 (continued)

Source material	Gene	Ch No.	Nature of resistance	References
Oryza sativa sp. *indica* (cv. TN 1)	*Xa14*	4L	Highly resistant to Philippine race 5	Bao et al. (2010), Yuan et al. (2010), Chattopadhyay et al. (2017)
Oryza sativa sp. *japonica* (cv. M41)	*Xa15*	NII	Provide maximum resistance to Japanese races	Gnanamanickam et al. (1999), Chattopadhyay et al. (2017)
Oryza sativa sp. *indica* (cv. Tetep)	*Xa16*	NI	Dominant and give resistance to Japanese isolates like J8581 and H8584	Noda and Ohuchi (1989), Oryzabase (2011), Chattopadhyay et al. (2017)
Oryza sativa sp. *japonica* (cv. Asominori)	*Xa17*	NII	Dominant and give resistance to Japanese isolates like J8513	Ogawa et al. (1989), Oryzabase (2011), Chattopadhyay et al. (2017)
Oryza sativa sp. *japonica* (cv. Toyonishiki)	*Xa18*	NI	Not effective against Asian strains but successful against African and Burmese strains	Noda et al. (1996), Gonzalez et al. (2007), Oryzabase (2011), Chattopadhyay et al. (2017)
Oryza sativa sp. *indica* cv. XM5	*Xa19*	NI	Resistant to utmost Philippine races	Taura et al. (1991), Chattopadhyay et al. (2017)
Oryza sativa sp. *indica* cv. XM6	*Xa20*	NII	Resistant to entire Philippine races	Taura et al. (1992a), Chattopadhyay et al. (2017)
Wild rice (*O. longistaminata*)	*Xa21*	11L	Provide widespread resistance at post-seedling stages to Indian and Philippine races	Song et al. (1995), Chattopadhyay et al. (2017)
Oryza sativa sp. *japonica* (cv. Zhachanglong)	*Xa22* (t)	11	Provide maximum resistance to 16 different strains from Japan, China, and the Philippines	Lin et al. (1996), Wang et al. (2003), Chattopadhyay et al. (2017)
Oryza rufipogon (wild rice)	*Xa23*	11L	Provide resistance to several Chinese, Philippine, and Japanese races	Wang et al. (2014), Chattopadhyay et al. (2017)
Oryza sativa sp. *indica Aus-boro line* (cv. DV86)	*Xa24*	2L	Dominant and provide resistance to several Chinese, Philippine, and Japanese races	Mir and Khush (1990), Wu et al. (2008), Khush and Angeles (1999), Chattopadhyay et al. (2017)
Oryza sativa sp. *indica* (cv. HX-3)	*Xa25a* (t)	4L	Provide resistance to several Chinese, Philippine, and Japanese races	Gao et al. (2001, 2005), Chattopadhyay et al. (2017)

(continued)

Table 2 (continued)

Source material	Gene	Ch No.	Nature of resistance	References
Oryza sativa sp. *indica* (cv. Minghui 63)	*Xa25*b (t)	12	Give race-specific resistance to Philippine races	Chen et al. (2002), Liu et al. (2011), Chattopadhyay et al. (2017)
Oryza sativa sp. *indica* (cv. Minghui 63)	*Xa26*	11L	Give broad-spectrum resistance to Philippine and Chinese races	Yang et al. (2003), Sun et al. (2004), Chattopadhyay et al. (2017)
Oryza minuta (wild rice)	*Xa27* (t)	6L	Give broad-spectrum resistance to strain no. 27	Gu et al. (2004), Wang et al. (1996), Chattopadhyay et al. (2017)
Oryza sativa sp. *indica* (cv. Lota Sail)	*Xa28*	NI	Recessive gene	Lee et al. (2003), Chattopadhyay et al. (2017)
Oryza officinalis	*Xa29* (t)	1	Dominant resistance to various races	Tan et al. (2004), Chattopadhyay et al. (2017)
Oryza rufipogon germplasm (Y238)	*Xa30* (t)	11L	Not clear	Jin et al. (2007), Chattopadhyay et al. (2017)
Zhachanglong	*Xa31* (t)	4L	Give resistance against the strain OS105 but susceptible to Px061	Wang et al. (2009), Chattopadhyay et al. (2017)
Oryza australiensis	*Xa32*	11L	Resistant to strains P1 (PXO61), P4 (PXO71), P5 (PXO112), etc. but susceptible to P2 (PXO86) and P3 (PXO79)	Zheng et al. (2009), Chattopadhyay et al. (2017)
Wild rice (*Oryza nivara*); (Acc. No. 105710)	*Xa33*	7	Provide broad-spectrum resistance	Kumar et al. (2012), Chattopadhyay et al. (2017)
Oryza sativa sp. *indica* (cv. BG1222)	*Xa34* (t)	1	Highly resistant to the Chinese race V	Chen et al. (2011), Chattopadhyay et al. (2017)
Oryza minuta (Acc. No. 101133)	*Xa35* (t)	11L	Dominant and give resistance to PXO61, PXO112, and PXO339	Guo et al. (2010), Chattopadhyay et al. (2017)
C4059	*Xa36* (t)	11L	Not known	Miao et al. (2010), Chattopadhyay et al. (2017)
Oryza nivara (acc. IRGC 81825)	*Xa38* (t)	4L	Resistant to all the races ubiquitous to northern states of India	Vikal et al. (2007), Cheema et al. (2008), Bhasin et al. (2012), Chattopadhyay et al. (2017)

(continued)

Table 2 (continued)

Source material	Gene	Ch No.	Nature of resistance	References
FF329	*Xa39* (t)	11	Provide broad-spectrum resistance to bacterial blight	Zhang et al. (2015), Chattopadhyay et al. (2017)
Oryza sativa sp. indica line IR65482-7-216-1-2	*Xa40* (t)	11	High levels of resistance to entire Korean races	Kim et al. (2015), Chattopadhyay et al. (2017)

Ch, chromosome number; NI, not identified

8 Genes and Techniques Employed to Rice Research

Various techniques have been utilized to produce stable transgenic rice plants in the market till date. However, transformation through *Agrobacterium tumefaciens* has been accepted as a routine technique in most of the laboratories (Toki et al. 2006; Delteil et al. 2010). Through this technique, efficiency of different rice varieties like japonica, indica, etc. has been improved sufficiently (Delteil et al. 2010). Later on, marker-assisted selection techniques were familiarized to the breeders for crop improvement (Ballini et al. 2009; Delteil et al. 2010). White and Yang (2009) describe the specific roles of major *Xa* resistance genes which confer the resistance against *Xanthomonas*. According to Peng et al. (2015), *Xa21* was the most prime gene in bacterial blight resistance which is also connected to signaling pathways. Other genes such as OsBRR1 and OsWAK1 were shown to be helpful in coding for receptor-like proteins and provide basal resistance to rice plant (Delteil et al. 2010). Moreover, genes which are utilized for the making of transgenic rice are basically related to defense signaling and transcription factors including XB15, OsPLDB1, OsDR8, SPL18, SPL11, PACK1, OsGAP1, and OsRac1 (Delteil et al. 2010). Moreover, few PR genes out of 14 found in rice have been overexpressed (Van Loon et al. 2006; Delteil et al. 2010).

In a recent study on RNA-Seq showed that rice cultivar IRBB61 containing *Xa7* when infected with *Xanthomonas oryzae* at high temperature stress, gives protection against the pathogen and was not dependent on salicylic acid pathway but related to abscisic acid (Cohen et al. 2017).

9 Future Goal and Conclusion

To find out the major constraints in rice production, it has been observed that bacterial blight has huge effect on yield loss. Though various approaches are available to control this disease, nowadays, the cultivation of resistant varieties is considered as the most economical tool. Many researchers till date working with the disease to understand the detailed molecular mechanism of pathogenesis. To protect rice from this devastating pathogen, it is an urgent requirement to identify and

characterize a large number of defense genes. Marker-assisted breeding and various transgenic approaches can be taken as potential tools to protect rice against this disease. Identification, characterization, and functional analysis of resistant genes can open the downstream signaling pathway associated with this disease. That may be altered at desired steps to check the efficacy of the pathogen. If we think in other way, application of various biotic and abiotic elicitors can also be taken as a tool to induce innate immunity against this disease. Application of newer genome editing tool as CRISPR against the disease can also be accepted as future area of research. Many transcriptomic, proteomic, and metabolomic approaches can be taken as tools to draw the defense signaling network.

To sum up at this point, our major goal is to protect rice plant from this pathogen. To achieve this goal and to secure our food, further research works are required for better understanding of pathogenesis and identifying probable easy tool to combat against this disease.

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Genetic Engineering of Cultivated Rice for Viral Resistance

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Abstract

Rice is one of the most widely cultivated and consumed staple foods for more than half of the global population. Viruses that infect rice can lead to huge losses and thus a serious impediment to rice cultivation. Development of strategies that are efficient enough to control these viruses has become the need of the hour. The rice-infecting viruses are transmitted by insects, mainly leafhoppers and plant hoppers, which can migrate over long distances. Genetic modification can pave way for plants that are resistant to virus attacks. Out of several developed methods, RNA silencing is the most promising one. However all RNAi constructs are not equally effective in conferring virus resistance. Some results show complete resistance, whereas some constructs slow down the appearance of symptoms. There are also possibilities that the construct may even fail to confer resistance. The success can be ensured by identifying appropriate viral gene. Hence with proper protocols, transgenic rice can lead to stable production of this staple food crop.

Keywords

Coat protein gene · Genome editing · Rice viruses · RNA interference · Viral resistance

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1 Introduction

Recent advancement in bioengineering improves the production of quality rice varieties, but still there is a vast number of drought, and disease-affected rice varieties need to be analyzed. Production of genetically engineered rice plants witnesses enhanced resistance against various viral, fungal diseases, and detrimental insects. Such developments appear to be most advantageous in rice genetics and conservation studies. The concept of resistance-derived pathogen interaction and the expression of several viral genes are proficient in minimizing various viral infections (Bajaj and Mohanty 2005). Various genetic, molecular, and genomic tools and techniques have been developed during the past two decades for producing virus-resistant rice plants (Fig. 1). Application of viral genes to converse resistance derived from pathogen against rice crops is a prominent method. The RNA interference (RNAi), also familiar as RNA silencing, is the efficient molecular approach to confer plant viral resistance on respective crop varieties (Sasaya et al. 2014). The rice cultivars indica and japonica are known as most popularly grown crop varieties in the world responsible for diverse genetic manipulations. Presently, available protocols for genetic transformation are tedious with less efficiency of transformation (Sahoo et al. 2011).

2 Viral Diseases in Rice

Rice (*Oryza sativa* L.) is one of the popular grain crops cultivated in most regions of the world. Asia alone contributes 90% of the total global rice production (Normile 2008). The loss of rice yield caused by viruses is enormous. It was necessary to safeguard food security for unabating population growth and diverse virus populations that damage rice plants. The rice viral agents comprise several viruses with double-stranded RNA (dsRNA) mainly, rice dwarf virus (RDV), rice black-streaked dwarf virus (RBSDV), and rice ragged stunt virus (RRSV); negative-sense single-stranded RNA viruses such as rice stripe virus (RSV) and rice grassy stunt virus (RGSV); a double-stranded DNA virus (dsDNA) mainly tice tungro

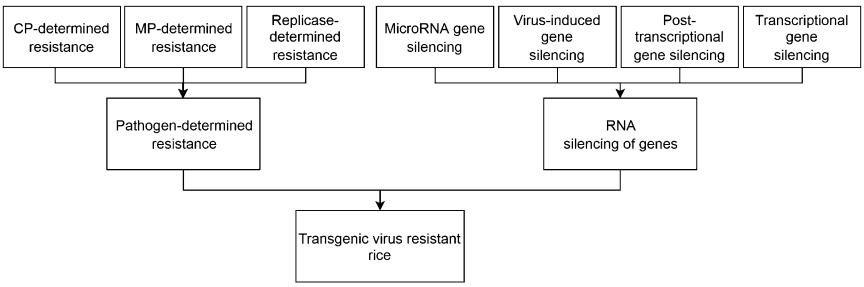

Fig. 1 Transgenic strategies used in production of virus-resistant rice plants (adapted and modified from Kyrychenko and Kovalenko 2018)

Table 1 Viral rice diseases and causative agents

Causative agent	Abbreviation	Disease	References
Rice grassy stunt virus	RGSV	Grassy stunt	Sarma et al. (2010)
Rice stripe virus	RSV	Rice stripe disease	Lijun et al. (2003)
Rice black-streaked dwarf Fijivirus	RBSDV	Black-streaked dwarf disease	Bottrell and Schoenly (2012)
Black-streaked dwarf virus	SRBSDV	Rice dwarf disease	Cuong et al. (2009)
Rice gall dwarf virus	RGDV	Rice gall dwarf disease	Chen et al. (2013)
Rice transitory yellowing virus	RTYV	Transitory yellowing disease	Hsieh and Roan (1967)
Yellow mottle virus	RYMV	Rice yellow mottle	Afolabi et al. (2009)
Rice necrosis mosaic virus	RNMV	Rice necrosis mosaic	Ghosh (1980)
Rice hoja blanca virus	RHBV	Hoja blanca	Morales and Niessen (1983)
Barley yellow dwarf virus	BYDV	Giallume	Amici et al. (1978)
Rice tungro bacilliform virus	RTBV	Tungro	Sarma et al. (2010)
Rice ragged stunt virus	RRSV	Ragged stunt brown	Sarma et al. (2010)

bacilliform virus (RTBV); and a positive-sense single-stranded RNA virus, rice tungro spherical virus (RTSV) (Sasaya et al. 2014). Most of rice viral pathogens are mainly disseminated by various plant hoppers and leafhoppers, and few of them multiply in insect hosts and transovarially transmitted that result in uncontrolled diseases management (Hibino 1996). List of rice viral diseases and the causal viral agent is given in Table 1.

The proficient rice transgenic plants are produced using two alternative strategies. One employs the natural potential of *Agrobacterium tumefaciens* to transfer gene of interest from a plasmid into the host plant genome (Hayakawa et al. 1992). The other way is a group of molecular techniques collectively known as direct DNA transfer, which consists of popular techniques mainly microinjection (Crossway 1989), protoplast-mediated transformation by polyethylene glycol [PEG] or calcium phosphate [$Ca_3(PO_4)_2$] (Datta et al. 1990; Cooley et al. 1995), electroporation (Zhang et al. 1988), particle bombardment (Christou et al. 1991), and silicon carbide whisker transformation (Nagatani et al. 1997). Further, some other transformation methodologies have also been modified and reviewed (Nandi et al. 2005). The main similarity among direct DNA transfer techniques is that external physical or chemical factors mediate the DNA delivery into cell (Kohli et al. 2003). The coat protein (CP)-mediated gene transfer is also a popular transformation technique used in rice plants mainly for rice stripe virus, introduced into two rice varieties of japonica by electroporation of protoplasts. The resultant transgenic plants expressed the CP at high levels (up to 0.5% of total soluble protein) and exhibited a significant level of resistance to virus infection (Hayakawa et al. 1992). Rice tungro disease is the most severe viral disease of rice. It is caused by infection from two viruses. This disease is caused by RTBV, while RTSV assists the transmission of viruses by

Fig. 2 Non-transgenic control plants (C) and transgenic rice progenies of line RTBV-O-Ds2 (T) with panicle emergence (indicated by arrow) (from Tyagi et al. 2008)

vector green leafhopper, *Nephotettix virescens*. Earlier studies have characterized RTBV and RTSV genomes, and virus-resistant transgenic rice plants have been produced using the CP-mediated resistance strategy (Fig. 2). Another strategy for producing virus resistant plants is the replicase-mediated resistance (Rep MR) strategy (Palukaitis and Zaitlin 1997). Huet et al. (1999) produced rice transgenic plants exhibiting the RTSV replicase gene in sense as well as in antisense orientation. The most preferably used reporter genes in rice transformation are the β-glucuronidase (GUS) (Jefferson et al. 1987) and green fluorescent protein (GFP) (Chalfie et al. 1994) as well as other reporter genes such as firefly luciferase (LUC); maize anthocyanin genes have been used (Tyagi et al. 2004). Though there are many modern biotechnological tools available for genetic transformation of rice, no such proficient technique for transgenic rice production has been developed till today.

3 Coat Protein-Mediated Resistance to Rice Virus Diseases

Rice diseases are the most crucial limiting factor inhibiting quality food productivity and sustainable conservation. Genetic technologies have provided novel strategies to enhance resistance in rice varieties. Improvement of viral disease resistance can

successfully be attained by producing genetically modified rice plants with collective gene expression responsible for coding viral coat proteins (CPs), repression of insect vectors, and expression of RNAi constructs. The advancement in plant genetic transformation has emerged as possible breakthrough in selection and introduction of genes into host plants to develop novel phenotypes. Some potential genes introduced preliminarily for fundamental studies, while other genes have subjected to their potential in developing of novel rice plants (Fargette et al. 2006; Zhang et al. 2009). The various plant virus genes, including those encode CPs of virus, have known to be more useful in improvement of virus-resistant rice varieties. The CP-mediated resistance was used to understand resistance caused by genomic insights of virus coat protein in transplants. Expression of viral CP genes in transgenic rice plants can lead to virus resistance by the interference of either transcript or protein with virus infection. Rice tungro disease is one of the crucial rice viral diseases, which limits productivity and stunt growth in major rice varieties. The disease is mainly caused by combined action of the RTSV and RTBV. The RTSV is responsible for protein supplements for virus transmission, while RTSV encodes symptom development. Recently, Malathi et al. (2019) have developed RNAi gene containing highly stable partial sequences of CP 3 gene of RTSV. The transgenic rice varieties were improved in background of japonica rice varieties Taipei-309 through *Agrobacterium*-mediated transformation. The transgene exhibited potential inheritance examined by PCR and Southern hybridization analyses. These genes expressed high-resistant phenotypes against tungro disease, and it was proved that CP3 of RTSV is a key target gene for the development of RNAi mediated tungro disease resistance in rice varieties.

The rice production in Africa is majorly affected by rice yellow mottle virus (RYMV) which is mechanically transferred by insect vectors (Fauquet and Thouvenel 1977). The development of disease-resistant rice plants would be crucial initiative to control major viral diseases and by in terms of high rice production. Kouassi et al. (2006) have developed transgenic variety of japonica TP309, to express RYMV CP genes under control of ubiquitin promoter. Eighty percent of the independent transgenic lines analyzed to confirm the presence of CP gene sequences. The genetically modified plants were analyzed for expression of RYMV gene, and it was observed that majority of plants having antisense nucleic acid sequences of CP genes and untranslatable CP mRNA showed a little delayed in accumulation of virus compared with non-trans TP309 plants.

Lentini et al. (2003) worked on the rice hoja blanca virus (RHBV) affecting rice yield in northern South America and Central America. This was the first report on transgenic resistance to RHBV and transformation of an indica rice variety from the Latin America. Rice with the RHBV nucleocapsid protein (N) gene had a notable reduction in the disease development. Several reactions were analyzed from susceptible to completely resistant plants. In a similar way, Zhou et al. (2012) investigated on rice stripe disease (RSD) in the rice-growing temperate areas, caused by widely RSV. They developed RNAi construct containing disease-specific protein sequences and CP gene from RSV. The RNAi construct genes were transferred into the two important japonica varieties, namely, Guangling Xiangjiang and Suyunuo, to

develop potent resistance against the rice stripe disease. The homozygous offspring of rice cultivar such as T5 and T7 generations with RNAi constructs exhibit efficient resistance to the viral infection. RT-PCR analysis suggested that virus replication of the CP in transgenic plants was successfully inhibited. The excellent agronomic characters of these two rice varieties, such as good quality and high yield, were maintained. Suppression of viral infection using RNAi promises novel and significant strategy for minimizing viral disease in rice crops. Shimizu et al. (2011) also investigated about RSV which has major concerns in temperate regions of East Asia. They mainly focused the selection of target gene sequences for RNAi and analyzed the effects of potential target sequences in each of genes in RSV genome using transgenic rice plants that exhibited a set of inverted repeat constructs. In another study, CP genes of RSV were introduced into the two japonica rice varieties by protoplasts electroporation. As a result, subsequent plants exhibited CP at higher levels of up to 0.5% of the total soluble protein and showed the significant virus resistance. These studies suggested that CP-mediated resistance to the virus disease can further be extended to the grains, cereals, and virus transmittance by insect vectors (leafhopper) (Hayakawa et al. 1992).

The production of genetically modified rice plants with potential S8 genes is crucial step in studying functional and phenotype aspects of RDV genes. The eighth largest segment of the RDV was derived from a RDV Fujian isolate. Then it was cloned into pTrcHis A vector for the expression in *E. coli* and further into pE3 vector for transformation. The callus from rice embryos subjected for analysis and obtained regenerated plants after the bombarding of target site with pE3R8 plasmid having RDV S8 gene and neomycin phosphotransferase II marker gene. The southern blotting ensures integration of RDV S8 gene into genome of rice, and further expressions of outer CPs in *E. coli* and plants were analyzed by Western blotting technique (Zheng et al. 1997). CP genes (CP1, CP2, and CP3) of RTSV were also introduced efficiently to japonica and indica rice to increase resistance. Plants from primary transformations were investigated to viral inoculation through leafhoppers. It was the first report of CP-mediated resistance against a virus that owns more than one CP gene from similar virus (Sivamani et al. 1999; Yoshii et al. 2009).

4 RNA Interference-Mediated Resistance to Rice Virus Diseases

Viruses infecting rice are a major threat to its cultivation causing huge yield losses. Insect vectors such as leafhoppers and plant hoppers are responsible for transmitting rice viruses over long distances. To protect crops from virus infection, resistance carrying genes from various natural sources can be exploited. Genetic engineering of rice cultivars can help to solve the problem of viral diseases (Sasaya et al. 2014). RNAi is a promising technique to confer virus resistance to rice cultivars (Shimizu et al. 2013). It is a gene-silencing strategy that is triggered by dsRNAs. The process involves an enzyme called Dicer. This enzyme cleaves double-stranded RNA into small interfering RNA (siRNAs) whose length varies from 21 to 24 nucleotides.

These small interfering RNAs are incorporated into a complex called RNA-induced silencing complex that mediates degradation of complementary target mRNA. Therefore rice plants can be conferred with resistance against viruses via expressing virus specific double-stranded RNA (Sasaya et al. 2014). RNA interference constructs that aim different viral genes vary in their effectiveness in preventing virus infection (Shimizu et al. 2009) (Figs. 3 and 4).

4.1 Resistance Against Reoviruses in Rice

There are mainly five rice-infecting reoviruses, namely, RDV, RBSDV, rice gall dwarf virus (RGDV), RRSV, and southern rice black-streaked dwarf virus (SRBSDV). Resistance against RDV has been developed in rice plants using RNA interference constructs that target viral genes code for proteins such as Pns6, P8, and Pns12. The extent of proposed resistance varied, but there was no difference in the growth or morphology of the plants. It has also been reported that NS10 gene from RRSV and P9-1 gene from SRBSDV are promising target genes (Sasaya et al. 2014).

4.2 Resistance Against Tenuiviruses in Rice

Tenuiviruses that mainly affect rice are rice hoja blanca virus (RHBV), rice grassy stunt virus (RGSV), and rice stripe virus. These are transmitted by plant hopper *Tagosodes orizicolus*, brown plant hopper and small brown plant hopper, respectively. Transgenic rice plants have been produced by introducing RNAi constructs of various RSV genes. Plants incorporated with RNA interference trigger constructs of viral genes for pC3 and pC4 proteins exhibited immunity to viral infection. Morphology and growth pattern of the transgenic and non-transgenic rice cultivars remained the same. Similarly, rice plants incorporated with RNA interference trigger plasmids for RGSV genes for pC5 and pC6 which were asymptomatic (Sasaya et al. 2014). In another study three transgenic rice cultivars were generated by incorporating an inverted repeat construct that aims nucleocapsid protein of the RSV (Li et al. 2016).

5 Plant Response and Field Performance of Virus-Resistant Rice Varieties

Rice as one of the important food crops in the world is under constant threat of infection by biological agents like fungal, viral, and bacterial agents and also climatic and soil factors, among which diseases caused by virus are more frequent (Michel et al. 2008; Wang et al. 2017). Diseases caused by viruses threaten the crop production especially in tropical areas of developing countries where there are less crop-free seasons and crops are extensively affected by viruses which are transmitted by vectors from wild and cultivated crop varieties (Kreuze and Valkonen 2017).

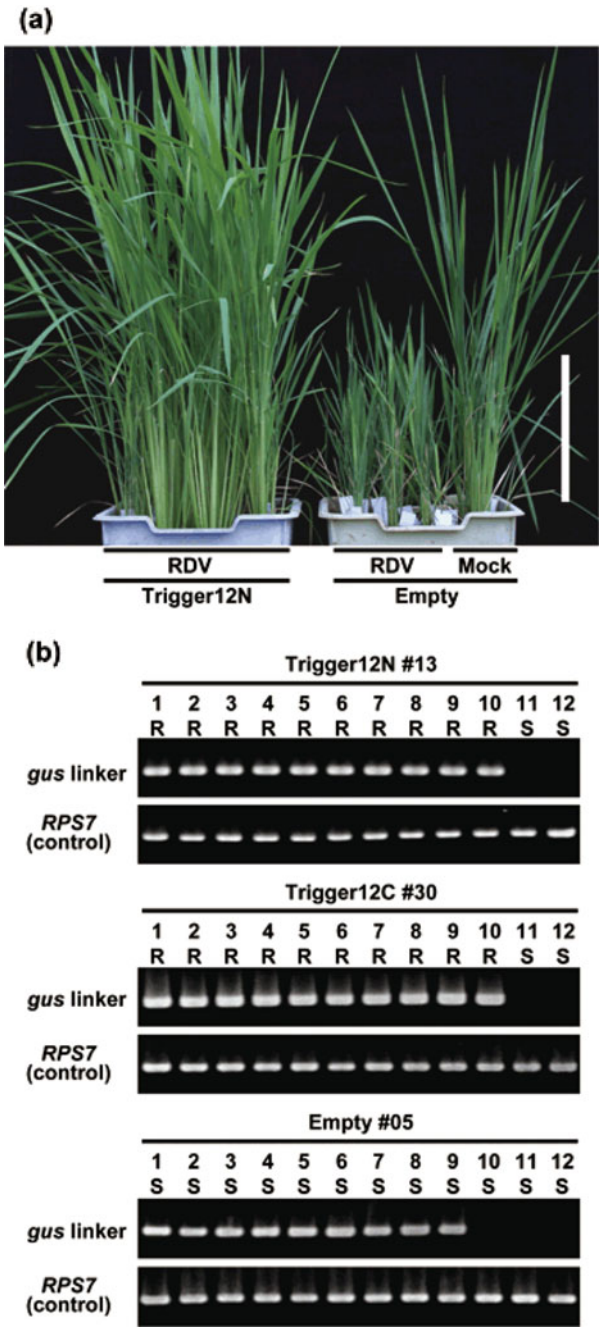

Fig. 3 Self-fertilized transgenic rice plants (T_1) with highly enhanced resistance to rice dwarf virus (RDV) by Pns12-specific RNAi: (**a**) RDV-inoculated transgenic T_1 healthy (left) and susceptible rice plants with empty vector as control and mock-inoculated plant (right) [Bar = 20 cm]; (**b**) correlation between transgene inheritance and infection susceptibility by RDV (lanes 1–12: individual progeny of transgenic rice plants with their response to infection; *R* resistance, *S* symptoms were apparent) (from Shimizu et al. 2009)

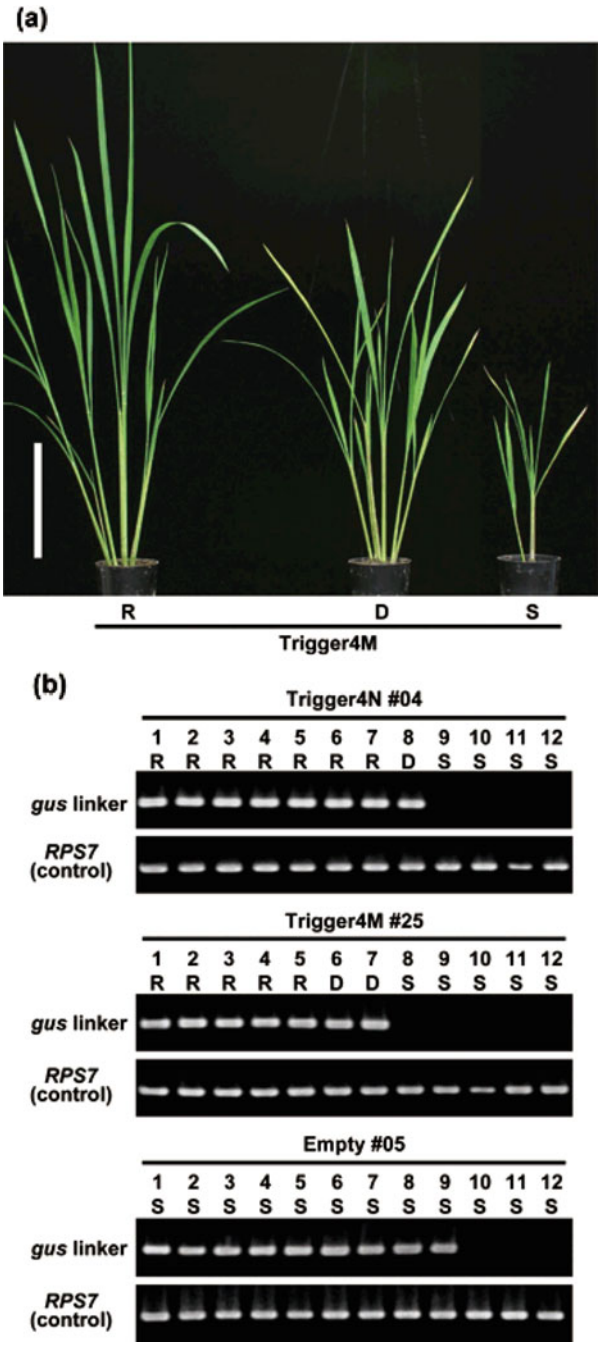

Fig. 4 Self-fertilized transgenic rice plants (T_1) with imperfectly enhanced resistance to rice dwarf virus (RDV) by Pns4-specific RNAi: (**a**) RDV-inoculated transgenic T_1 rice plants—resistant (R), delayed symptoms (D), and susceptible (S) [Bar = 20 cm]. (**b**) Correlation between transgene inheritance and infection susceptibility by RDV. (Lanes 1–12: individual progeny of transgenic rice plants with their response to infection AS resistant (R), delayed symptoms (D), and susceptible (S) (from Shimizu et al. 2009)

RBSDV, RSV, and RDV that attacks rice crops are transmitted by plant hoppers and leafhoppers. RYMV which belongs to the genus *Sobemovirus* damages rice by causing rice yellow mottle disease (Longue et al. 2018). Rice tungro disease is caused by RTSV and RTBV (Ladja et al. 2016). Some of the rice-resistant varieties against viral diseases have been given in Table 2.

RRSV and SRBSDV are predominant in Japan, China, and Vietnam. Wang et al. (2017) conducted field study in Yunnan, China, to analyze the resistance of certain rice varieties to RRSV and SRBSDV. Parameters like acre yield, load of virus, quantity of insects per 100 clusters, and incidence of plants infected were used to evaluate the resistance. Among the tested varieties, Liangyou2186 (L2186) and Zhongzheyou1 (Z1) showed highest rate of yield, and IIY58, N5Y39, Z1, and Y1 showed highest antivirus features against SRBSDV. Comparative label-free shotgun LC-MS/MS (liquid chromatography tandem-mass spectrometry) was used to understand the proteomics of the tolerance of rice toward SRBSDV (Fig. 5).

Studies on different resistant varieties of RYMV have shown that resistance exhibited by each variety toward the virus is different. Field performance conducted

Table 2 Virus-resistant varieties of rice developed using genetic, molecular, and transgenic approaches

Virus	Resistant rice variety	References
Rice yellow mottle virus	Tog7291,Tog5672, Tog5681,Tog5674 (*Oryza glaberrima*), Bekarosaka, Gigante (*Oryza sativa*), IAC25, IRAT13, Ngovie, 056, Moroberekan	Longue et al. (2018), Okioma and Sarkarung (1983)
Rice tungro spherical virus and rice tungro bacilliform virus	IR20, IR26, IR30 IR 36, IR28, IR29, IR34, IR36, IR38, IR40, Utri Merah, Tiempo Kitjik, TKM 6, ARC 10312, ARC 12596, Habiganj Deep Water, Tukad Petanu, Inpari 7 Lanrang	Khush et al. (2004), Ladja et al. (2016)
Rice ragged stunt virus	Utri Rajapan	Panda et al. (1984)

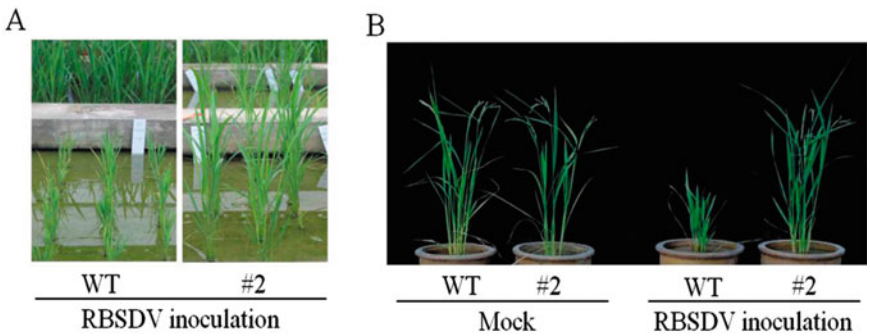

Fig. 5 Transgenic rice plants with rice black-streaked dwarf virus (RBSDV) resistance, 30 days post-inoculation (**a**) and 60 days post-inoculation (**b**). WT, wild-type Kitaake; #2, hpRNA transgenic rice plant; mock, mock inoculation (as negative control) (from Wang et al. 2016)

showed that Moroberekan which is a japonica type is the highly resistant variety that can be grown in lowlands, while WAT316 and WITA9 lowland varieties which belong to indica group were less resistant compared to Moroberekan. Upland varieties like IDSA46, IDSA76, and IDSA62 belonging to japonica type also showed tolerance toward RYMV. After inoculation Moroberekan did not show any symptoms of virus infection, whereas other varieties showed slight symptoms (Michel et al. 2008). RTSV and RTBV which cause rice tungro disease are transmitted by green leafhoppers. Green leafhoppers can independently transmit RTSV whereas it can transmit RTBV only when RTSV is present (Shim et al. 2015). Hibino et al. (1990) conducted a study on certain rice varieties that are resistant to rice tungro virus to understand the level of resistance. Plant resistance was studied using mass inoculation and test-tube inoculation methods. Utri Merah variety showed the maximum level of resistance compared to other varieties which were studied.

6 Conclusion

Since the first announcement of transgenic rice crops LLRice60 and LLRice62 conferring herbicide resistance in 2000, there have been notable breakthroughs both in laboratories and agricultural fields. Concerning to productive field applications, antiviral resistance has become essential traits for most of commercialized rice crops. However, novel technologies which enable rapid identification of potential immune receptor genes have improved viral resistance in most of the rice varieties. The use of CP genes and RNAi is the most potential and promising technique to confer strong resistance against rice viruses and has substantially expanded. While improving transgenic rice plants with strong resistance against rice viruses, it is important to target causative viral genes that play an important role in viral infection and proliferation at an early stage of viral replication. Advancements in bioengineering tools mainly targeted gene therapy and targeted genome editing approaches that are anticipated to play a key role in inducing wide range resistance against both viral and non-viral rice pathogens in the foreseeable future. Additionally, increasingly efficient and versatile genome editing techniques like CRISPR-Cas may provide targeted alterations of endogenous genes for viral resistance in rice.

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Genomics and Genetic Engineering for Polyamine-Mediated Tolerance of Rice Against Pathogen Infection

Dew Biswas, Tania Ghatak (Chakraborty), Anuradha Mukherjee, Samapika Nandy, Devendra Kumar Pandey, and Abhijit Dey

Abstract

A specialized group of aliphatic polycations is referred to as polyamines distributed ubiquitously in prokaryotic and eukaryotic organisms. Free polyamines as well as conjugated polyamines are found in living cells. In plants, polyamines are synthesized from arginine via two alternative pathways. Plants have well-organized transport system for polyamine conduction. Among the wide range of physiological functions of polyamines, stress response is an important one. Plants have evolved a variety of mechanisms to recognize distinct stimuli for the alteration of the gene expression in response to different types of stresses (Hatmi et al. Osmotic stress and ABA affect immune response and susceptibility of grapevine berries to gray mold by priming polyamine accumulation. Front Plant Sci 65:75–88. https://doi.org/10.3389/fpls.2018.01010, 2018). Often, partially overlapping sets of responses are activated in different stress conditions, and several points of crosstalk exist between abiotic and biotic stress responses. Rice, an important food grain of South Asian countries, has been confronted with several stresses in the field of agriculture. Destructive pathogenic attack is one of the major challenges for their survival and good production. Apart from the conventional chemical treatment, modulation of genes for polyamine biosynthesis and expression might be a worthwhile means of solution for this problem.

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Considering the preventive role of polyamines during microbial infection, scientists are now trying to develop resistant varieties with the help of genetic engineering. In the present review, some relevant aspects are talked over to find out some cues about the detailing of the respective gene expression regarding this particular issue.

Keywords

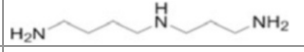
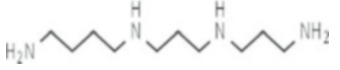
Polyamines · Aliphatic compounds · Glutamine · Glutamate · Transport · Stress tolerance · Gene study

1 Introduction

Polyamines are the distinctive category of organic compounds with more than two amine groups, showing prevalent distribution within the living organisms. These are aliphatic nitrogenous compounds produced by organisms during their metabolic activities (Roychoudhury and Das 2014; Chen et al. 2019). Low molecular weight linear polyamines are found predominantly in all types of life forms. They play pronounced roles in cell growth, membrane transport, ion channel modulation, senescence regulation, etc. Commonly, polyamines found in plant cells have two forms: free and conjugated. When free polyamines covalently combine with phenolic compounds and their derivatives through amide bonds, formation of conjugated polyamines takes place. In most of the cases, this reaction is catalysed by the action of transferase or transglutaminase. The phenolic compounds may be hydroxycinnamic acid, coumaric acid, caffeic acid or ferulic acid (Luo et al. 2009; Martin-Tanguy 1997). In higher plants the most commonly found polyamines are putrescine (Put), spermidine (Spd), spermine (Spm), thermospermine (Tspm) and cadaverine (Michael 2016). Chemical formulae and structures of those five types of polyamines are given in Table 1. Plant polyamines are involved in regulating several developmental processes like cell division, breaking dormancy, germination, bud development and fruit set together with plant response to various biotic and abiotic stresses (Paul et al. 2018).

Polyamines were first discovered by Antonie van Leeuwenhoek in 1678 as a crystalline substance in human semen sample. In 1791, Nicolas Vauquelin described those as phosphate salts of a cation and presented their insolubility in water and ethanol. Charcot (1853) published an article on crystallization of spermine phosphate. Boettcher assumed that the structural constituent of that substance was protein, and in 1865 he gave the name “spermatine”. In 1888, Landenberg and Abel used the word “spermine” for those organic compounds. In 1926, Otto Rosenheim determined the chemical structure of spermine. Brieger was credited in 1885 for the discovery of putrescine and cadaverine (Mendez 2017). In general, polyamines are a particular class of polymers possessing ionizable amine moieties (Wallace 2009).

Table 1 Chemical formulae and structures of five major polyamines in plant

Serial No.	Abbreviated name	Full name	Chemical formula	Chemical structure
1	Put	Putrescine	$C_4H_{12}N_2$	
2	Spm	Spermine	$C_{10}H_{26}N_4$	
3	Spd	Spermidine	$C_7H_{19}N_3$	
4	Tspm	Thermospermine	$C_{10}H_{26}N_4$	
5	–	Cadaverine	$C_5H_{14}N_2$	

Polyamines have effective roles in plants for stress adjustment as they regulate gene expression during elicitation of stress response (Paul and Roychoudhury 2017). They are engaged in contributing to cope with various abiotic stresses like drought, cold, salinity, etc. Often, biotic stress tolerance of plants can be correlated with the induction of genes involved in polyamine metabolism and higher level of polyamine production (Walters 2003). Pathogenic infection is one of the major sources of biotic stress of plants. Here, we will discuss mainly on polyamine metabolism under different types of pathogenic attacks on plants especially for rice along with their biosynthesis and transport mechanism and some other important aspects regarding genomics and genetic engineering for the improvement of stress tolerance.

2 Polyamine Biosynthesis in Plants

Free and conjugated polyamines are found in plants depending on the types of tissue and stages of development (Geny et al. 1997). Sometimes, adverse conditions like long-term water stress influence on the alteration in cellular-free polyamine concentration (Hura et al. 2015). Salt stress also induced to increase predominant polyamine (Put, Spd, Spm) pools (Roychoudhury et al. 2011; Do et al. 2014). Acid-soluble conjugated polyamines such as hydroxycinnamic acid amides interact with nucleic acids and regulate several physiological functions (Martin-Tanguy 1997). Polyamine conjugates are increased often in response to various types of biotic and abiotic stresses. According to the previous reports, biosynthesis of polyamines is almost conserved in all types of eukaryotes and prokaryotes with some minor differences (Minguet et al. 2008; Pegg 2009). Spermidine and spermine as well as their diamine precursor referred to as putrescine represented a highly conserved, evolutionarily important set of organic polycations which is involved in the modulation of a number of biological processes such as enzymatic activation, ionic balance, growth regulation, hormonal activities, cell cycle maintenance, etc. Plant-derived compounds glutamine and glutamate are the starting point of amine biosynthesis. There are two alternative pathways proposed by authors for polyamine biosynthesis in plants (Bagni and Tassoni 2001; Liu et al. 2006). The three polyamines Put, Spd and Spm are synthesized from arginine via ornithine by the enzymatic action of ornithine decarboxylase, mainly. On the other hand, those are synthesized from arginine via agmatine and N-carbamoylputrescine through the sequential activities of arginine decarboxylase, agmatineiminohydrolase and N-carbamoylputrescine amidohydrolase. Arginine decarboxylase was considered to be a contributor of first rate-limiting step, here. Participation of arginine and ornithine directly connected via citrulline relatively depends on tissue type, substrate concentration and physiological usage (Adiga and Prasad 1985). It was evident that putrescine (Put) biosynthesis depended on arginine decarboxylase and ornithine decarboxylase and other two amines called Spd and Spm were derived from it (Carbonell and Blázquez 2009). Tspm is an isomer of Spm, found in some species like *Thalassiosira pseudonana*, *Arabidopsis thaliana*, etc. (Knott et al. 2007). Another one polyamine, cadaverine, possesses similar chemical properties of

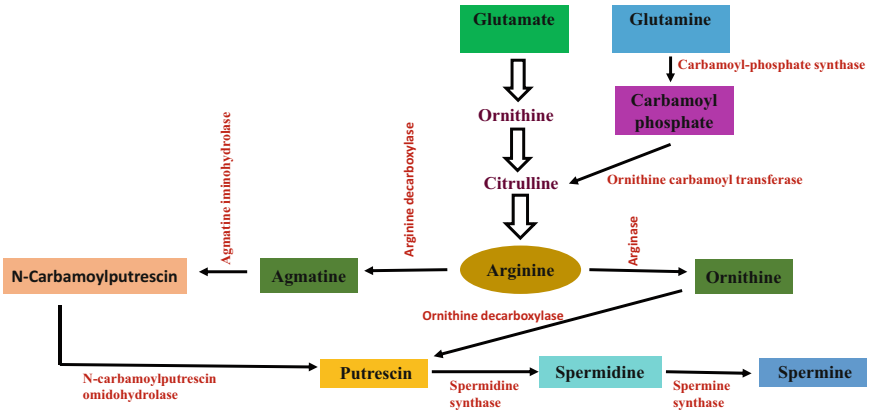


Fig. 1 Polyamine biosynthetic pathway in plant

putrescine (Gamarnik and Frydman 2008). Homoarginine-lysine decarboxylase mediates the synthesis of cadaverine (Adiga and Prasad 1985). A simplified representation of polyamine biosynthetic pathway is depicted in Fig. 1.

3 Transport of Polyamines in Plant

Naturally, plants face several challenges to survive in different types of stressed condition. Unlike animals they are not able to escape from this exploitation of genetic resources. Critical role of polyamine for scavenging tissue damage in stress has been suggested through several lines of research. Plant cells are able to synthesize polyamines according to their needs. In spite of this, cells are well equipped with efficient transport system to uptake exogenous polyamine compounds (Tanguy-Martin 2001). In general, polyamine transport depends on cellular requirement with respect to the entire development of plant (Fujita and Shinozaki 2014). Intracellular polyamine pool greatly depends on the respective mechanism of transport (Antonio et al. 1997). This also plays an important role in homeostatic regulation of polyamine level. Rapid uptake of Put and Spd by Ca^{+2} stimulation was reported in the protoplast of carrot (Antognoni et al. 1994). Energy-dependent, protein-mediated Put transport across plasma membrane was observed in maize root (Ditomaso et al. 1992). Fujita and Shinozaki have hypothesized RMV1 as a polyamine transporter in *Arabidopsis* as the plants overexpressing RMV1 displayed higher polyamine uptake activities in comparison with the control lines. They also identified LAT (L-type amino acid transporter) as one of the polyamine transporters in *Arabidopsis* (Fujita and Shinozaki 2014). Based on radiological investigation and phylogenetic analysis, Mulangi et al. (2012) marked a gene OsPUT1 as polyamine uptake transporter in rice (Mulangi et al. 2012). Active transport of polyamines in higher plants is significantly influenced by auxin and the amount of absorbed polyamines which is mainly stored in vacuoles. Study on long-distance transport has revealed that translocation of

polyamines throughout the plant body predominantly occurs via xylem vessels depending upon transpiration (Tanguy-Martin 2001). This effects on tissue development in both normal and stressed condition significantly as moving through the vascular system of plants. Export of polyamine and the probable reasons beyond are still not defined well. A few reports are stating something like putrescine exported during floral transition responding to specific signals (Tanguy-Martin 2001). Further study is needed to answer some unsolved questions in respective issues.

4 Polyamine Metabolism Under Biotic Stresses

Responding to different types of stresses, polyamine metabolism of plants is regulated by means of different mechanisms. Metabolism of polyamine in plant cell is presented here in a simplified diagram (vide Fig. 2). It was reported that abiotic stresses effected on assorted aspects of polyamine metabolism through diverse mechanisms of actions (Gupta et al. 2013). Likewise, biotic stresses also have significant impact on the metabolism of polyamine-based compounds produced inside the plant body. Gene expression for polyamine biosynthesis and their metabolism during pathogenic infection leads to variation in concentration of respective compounds in host plants (Walters 2003). Being a complex dynamic process, plant-microbe interaction usually promotes notable changes in polyamine metabolism of plant. Metabolic pathway may depend on the nature of microorganism and activation of plant defence mechanism that takes place (JimÃénez-Bremont et al. 2014).

It was reported that infection of barley by biotrophic fungus *Puccinia hordei* triggered an increase in Spd level. Accordingly, higher accumulation of polyamine biosynthetic plus catabolic genes was observed in resistant variety but not in susceptible lines (Walters 2000). Marini et al. (2001) reported that polyamine metabolism was activated in resistant NN line of tobacco along with the accumulation of Spd and Put, and those were not found in susceptible ones (Marini et al.

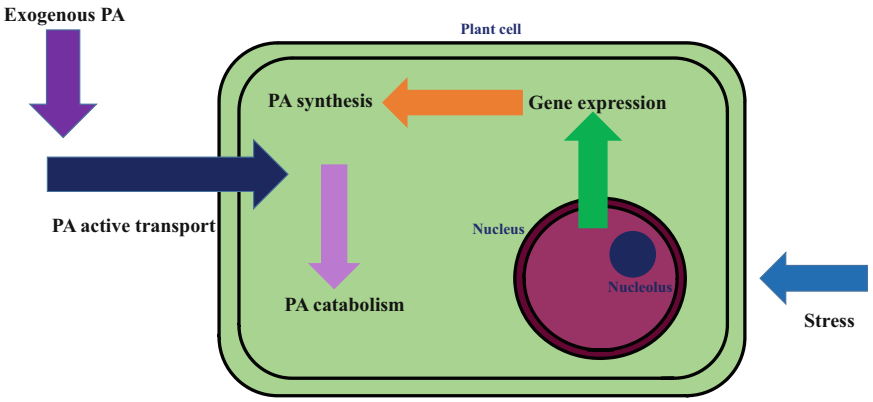


Fig. 2 Polyamine (PA) metabolism in plant cell

2001). Rapid induction of thermospermine synthase in response to *Verticillium dahliae* attack was seen in cotton, and that was not found in resistant group (Mo et al. 2015). Putrescine was accumulated in whole leaf tissue as well as in apoplast of tomato plant infected with *Pseudomonas syringae* as per the investigation of Vilas et al. (2018). Polyamine accumulation mainly depends on the induction of two principal biosynthetic enzymes arginine decarboxylase and ornithine decarboxylase. Not only the free forms but also the conjugated polyamines are accumulated as a very usual event in pathogenic infection (Walters 2003). Alteration in the host defence mechanism is mediated through polyamine oxidation mostly despite non-oxidized polyamines are also a part of cellular responses (JimÃénez-Bremont et al. 2014). Accumulation of peroxides as a result of polyamine metabolism seems to play a significant role in signal transduction in plant-pathogen interaction (Walters 2000).

5 Polyamines to Improve Stress Tolerance of Rice During Pathogenic Infection

Seed endosperm of *Oryza sativa* (rice) belongs to the family Poaceae and is the chief food substance throughout the Indian subcontinent, and around three billion of people as a whole use rice as main dietary supplement all over the world. There is a long history where different types of pathogenic infections like brown leaf spot disease of rice, bacterial blight of rice, tungro virus disease of rice, etc. greatly limited the crop yield. Application of chemical treatment mediates stoppage of progressive infection spreading. However, emergence of some chemical resistant pathogenic strains has made it more difficult to fight with those diseases, completely. On the other hand, overuse of chemical compounds in disease management may cause pollutant accumulation in the surrounding environment. Efforts are now given to control such destructive attacks by means of developing resistant variety through the modulation of endogenous polyamine pool using genetic engineering. Several studies have demonstrated that constitutive or inducible overexpression of several genes altered the endogenous level of polyamines in rice plant and enhanced plant tolerance to various stressed conditions (Hussain et al. 2011; Shi et al. 2014). Modulation of some other genes having active involvement in polyamine biosynthesis and metabolism was also considered to be the responsible one in stress management (Moschou et al. 2012). Some knockout mutants showing lower Put and Spm accumulation exhibited decreased stress tolerance. Downregulation or overexpression of apoplastic polyamine oxidase modulated stress tolerance of a number plant species (Cuevas et al. 2008; Tavladoraki et al. 2012). In 1998, Yamakawa et al. marked Spm as an inducer of viral pathogenic resistance and accumulation of pathogenesis-related proteins for self-defensive activity in plants (Yamakawa et al. 1998). Overexpression of arginine decarboxylase in transgenic plants caused considerable increase in agmatine, free polyamine fractions as well as the conjugated forms. The modification of polyamine biosynthetic pathway through this manner may alter the level of stress adaptation of many crops (Bassie et al. 2008;

Burtin and Michael 2015; Prabhavathi and Rajam 2007). Polyamines participate in vital developmental stages of higher plants including rice and also take part in increasing plant resistance to biotic stresses. It was suggested by Hussain et al. (2011) by the activation serine/threonine kinase from MAPK group polyamines elicit HR gene expression that leads to local cell death at the site of infection (Hussain et al. 2011). Polyamines activate diamine oxidase and polyamine oxidase, the two sources of hydrogen peroxides which are used during the lignification of cell wall and the programmed cell death signalling. In addition, hydrogen peroxide resists pathogenic growth inside the host, directly (Walters 2003). Polyamines mediate DNA stabilization/destabilization through binding interactions which influence the function of transcription factors and gene expression in infecting organisms. An evidence has been shown in a report indicating that the synthetic analogue of putrescine inhibited cysteine DNA methylase and caused significant growth retardation of pathogens (Walters 1997). Being associated with pectin polysaccharides, polyamines are involved directly in cell wall strengthening and in controlling of lignin deposition plus pH of the cell wall through which they reduce the smooth pathogenic invasion into the host cells (Angelini et al. 1993; D'Orazi and Bagni 1987). As per the discussion above, modification of the expression of endogenous polyamine biosynthetic and metabolic genes may be deployed in developing transgenic resistant varieties of rice following certain authentic principles based on genetic engineering and plant biotechnology.

6 Polyamines in Relation to Abiotic Stress Tolerance in Rice

Role of polyamines in abiotic stress response of rice is discussed briefly here to highlight their significant activity during unfavourable environmental condition, and this may give some hints regarding some common queries like does polyamine gene expression occur universally in all types of stresses, whether there is any correlation between abiotic stress response and biotic stress response or not, etc. In transgenic rice, tolerance to drought stress was implicated to the modulation of the polyamine biosynthetic pathway. Transgenic rice plants expressing *Datura stramonium adc* gene produced much higher levels of putrescine when exposed to drought stress, which in turn facilitated the synthesis of spermidine and spermine in order to protect rice plants from water deficit (Capell et al. 2004). It was suggested that rice plants must possess higher concentrations of free spermidine/free spermine and insoluble-conjugated putrescine for the survival in drought condition. Moreover, early accumulation of free polyamines was also indicated in combating water stress in rice (Yang et al. 2007). Exogenously applied polyamines enhanced drought tolerance in rice by promoting leaf water status, photosynthesis and membrane properties. Foliar application was suggested as more efficacious as compared to seed priming, and spermine was found to be the most effective polyamine meant for drought tolerance (Farooq et al. 2009). Saline resistance in rice plants was attributed to the highly enhanced levels of polyamines and decrease of diamines. In salt-tolerant AU1, Co43 and CSC1, high levels of spermidine and spermine were noted, while the putrescine

level was almost unaltered when exposed to salinity stress (Krishnamurthy and Bhagwat 1989). In a comparative study between the salt-sensitive M-1–48 rice and the salt-tolerant Pokkali rice, the reversal of salinity stress by exogenous polyamines was found to be more pronounced in the salt-sensitive cultivar (Chattopadhayay et al. 2002). *S*-Adenosylmethionine decarboxylase (SAMC) gene overexpression in rice was implicated to the rise in polyamine level in relation to sodium chloride-stress tolerance (Roy and Wu 2002). In various rice genotypes, an increase in arginine decarboxylase (ADC) activity with simultaneous synthesis of polyamines was noted during salt stress (Basu and Ghosh 1991). In rice cv. Rupsail seedlings, salinity resulted in higher accumulation of polyamines via enhanced arginine decarboxylase (ADC) activity (Basu et al. 1988). In chilling tolerance of rice seedlings, raise in abscisic acid (ABA) levels promoted arginine decarboxylase (ADC)-mediated putrescine synthesis (Lee et al. 1997). Under oxygen-deficit stress in rice, a remarkable enhancement of ADC was noted in the coleoptile and root of the rice seedlings (Reggiani et al. 1989). Both conjugated and free polyamines were found to be accumulated in higher amount in callus of the heat-tolerant rice cultivar N22 compared to that of the heat-sensitive rice cultivar IR8 exposed to high temperature. This increment in the polyamine level was attributed to the higher ADC and polyamine oxidase activities in the heat-tolerant rice cultivar (Roy and Ghosh 1996). In rice leaves, cadmium (Cd)-induced oxidative damage was reportedly reduced by polyamines via inhibiting H_2O_2 generation and reducing Cd uptake (Hsu and Kao 2007). Therefore, polyamines play a significant role as a stress modifier not only against the biotic stresses encountered by rice but also against an array of abiotic stresses.

7 Future Prospect and Conclusion

Recently, polyamines are considered as a significant outlook in genetic engineering as having a long history of research together with a multitude of physiological activities. Among these, their accumulation in response to plant-microbe interaction is an important one. They play a supreme role in shielding plant cells from stress-related damages. Understanding the significance of polyamine accumulation and its level of changes in response to different type of biotic stress is in progress. However, it is often difficult to identify their relevant contribution at the site of infection. It necessitates the study of the expression of sense and antisense sequences under the control of tissue-specific promoters regarding the events of polyamine biosynthetic pathway. Enrichment of research activities has presented that the response of polyamines during stress is possibly due to the altered gene expression of their biosynthesis and metabolism. Details of signal transduction of polyamines and crosstalk with other phytohormones are still not represented. Investigation on the RNA-based novel regulatory mechanism may provide some cues to divulge exact means of stress tolerance with respect to polyamine modulation together with signalling and correlation with plant growth regulators. Exploration of nature and functional characteristics of respective polyamine compounds might be a useful tool

in terms of comprehending the metabolic complexities. Examination of the existence of link between internal polyamine content and intercellular transport deserves keen attention from this point of view. Molecular mechanism behind polyamine accumulation including the biosynthetic genes and their transcriptional regulation network responding to pathogen-related stresses must be clarified also. Genome-wide expression analysis of plants under pathogenic infection may help to untie regulatory mechanism of polyamine metabolism under biotic stresses, specifically, and this may be utilized for generating transgenic resistant varieties of important crop plants using advanced biotechnological approaches.

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Genomics and Genetic Engineering of Rice for Resistance to Different Insect Pests

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Abstract

Adaptive behavior is one of the crucial salient features for any living organism with a great relevance toward resistance through generations against exposure to chemicals or any other material like pathogens or insect pest. Due to continuous exposure to insect pests and other pathogens, there are great total yield losses and depreciation in the quality as well as in the quantity of cultivated plants. In the current scenario, excessive use of chemical-based pesticides and synthetic fertilizers in agronomy results in soil fertility reduction. Intracellular bioaccumulation of pesticide resistance of insects and other plant pathogens has emerged globally. Several species of plants have reported to been transformed for resistance against insects and other pathogens including viruses, bacteria, fungi, and nematodes. Better understanding in the mechanism of resistance has unlocked new insights in the field of host-plant resistance. Genomics and genetic engineering may be used to improve natural rice (*Oryza sativa L.*) plant resistance to insect pests as can also be done through conventional crossbreeding among plants or varieties within *Oryza sativa L.* Gene transfer for nutritionally rational biosynthetic pathway has given new insight for executing metabolic machinery of the organism. In rice, several genes have been transferred for resistance to insect pests and other pathogens and also for abiotic stress. Furthermore, important specific target genes require significant progress for functional genomics in rice. The combination of genomics and genetic engineering provides targeted expression, and effective new genes should pave the way for improvement of *Oryza sativa L.*

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Keywords

Genomics · Resistant gene · Exposure to pesticides · *Oryza sativa* · Genetic engineering

1 Introduction

Globally, rice is one of the most rationally important foods; unfortunately, its production suffers significantly from insect pests, causing losses of billions of dollars, and extensive use of environmentally hazardous synthetic chemicals in the form of pesticides for their control. One of the significant major goals in the field of plant biotechnology is construction of genetically engineered cereals like wheat, rice, and maize with improved resistance against various diseases and harmful insects (Swaminathan 1982). The major damage to the crops due to virus borne pathogens and crops are prone to acquire virus infection directly or indirectly, and protection against viruses has considerably reduced the overall loss in many crops in terms of yield (Gasser and Fraley 1989; Vasil 1990). Previous report on transgenic tobacco plants that express tobacco mosaic virus (TMV) coat protein (CP) exhibited resistance against infection of TMV; this strategy has been widely used for protection against a number of viruses (Powell-Abel et al. 1986). Surprisingly, this strategy has not been applied to cereals, the most important group of plants globally, largely because reproducible transformation has not been available until recently. Rice stripe virus (RSV), which causes severe damage to rice in mainly Asian countries like Korea, Japan, Taiwan, China, and also the Commonwealth of Independent States (CIS), is a type member of the *Tenuivirus* group and is transmitted by the small brown planthopper, *Laodelphax striatellus*, in a persistent manner. RSV has four species of double-stranded RNA and four species of single-stranded RNA, which are designated RNAs 1–4 in order of decreasing molecular mass (Beachy et al. 1990; Gingery 1988). The single-stranded RNAs were predicted to be counterparts of the double-stranded RNAs. The CP of RSV was deduced to be encoded on the genome of RNA-3, which is confirmed through RNA-3 sequence analysis. In addition to the CP, the rice plants infected by RSV show a large amount of accumulation of ~20-kDa protein, called stripe disease-specific protein (SP) (Hayakawa et al. 1992).

The very first significant step for the development of insect-resistant rice is through introduction of a truncated gene of δ-endotoxin, *cryIA(b)* of *Bacillus thuringiensis* (*Bt*) that has specific biological activity against several insects into rice plant. Inducible form of *cryIA(b)* gene in rice in the coding sequence was extensively modified based on the codon usage of rice genes. Transgenic plants efficiently expressed the modified *cryIA(b)* gene at both mRNA and protein levels. Bioassays using R_2 generation plants with two major rice insect pests, striped stem borer (*Chilo suppressalis*) and leaf folder (*Cnaphalocrosis medinalis*), indicated that transgenic rice plants expressing the *CrylA(b)* protein are more resistant to these pests than untransformed control plants. Our results suggest that the *Bt* endotoxin

genes will be useful for the rational development of new rice varieties resistant to major insect pests.

Majority of studies on resistance of plants to sucking pests has concentrated on the role of semichemicals and secondary metabolites of plants as feeding deterrents. The feasibility of engineering transgenic plants to confer the ability to produce secondary metabolites has yet to be demonstrated, and the ability to do this on a routine basis for given secondary compounds is in the future due to the complexity and species specificity of the biochemical pathways involved—although this approach is now being addressed (Hallahan et al. 1992). For some insect pests, the expression of *Bacillus thuringiensis* (*Bt*) endotoxin genes in transgenic plants has been shown to be an effective means of control, although the long-term use of *Bt* may depend on devising suitable management strategies to delay the buildup of *BT*-resistant insect populations. However, sucking insects are not amenable to control by *Bt* bacteria, or toxins, at present, since no reported strain of Bt is effective against homopterous. To tackle the problem of producing transgenic plants with resistance to sucking pests, it was necessary to go back to insect bioassays. Products of genes that could be obtained reasonably easily, and which could be expressed in transgenic plants using existing technology, were assayed for their effect in artificial diet bioassay.

Identification certain biomarkers (specific proteins) against exposure to insecticides through Insect bioassays, where BPH feeds exclusively on the phloem and xylem saps of rice plants, with the phloem sap only providing a source of nutrients. An artificial diet system for this insect must thus mimic its natural foodstuff. A liquid diet formulation, containing sucrose, amino acids, and vitamins, is used; portions of this diet are enclosed in parafilm sachets (which can be put under pressure, to simulate the normal phloem pressure in the plant), and the insects feed by probing the parafilm and sucking the diet in the same way in which they normally probe plant tissues and suck phloem sap. The diet allows the insects to develop through several nymphal stages to adults quite successfully with survivals of more than 50%, but is not suitable for rearing successive generations of insects (Powell et al. 1993). Proteins with insecticidal properties toward BPH assay of a number of plant and other proteins against BPH in the bioassay system described above showed that the presence of an inert protein, such as ovalbumin, had no deleterious effects on survival, but some biologically active proteins were toxic (Powell et al. 1993). Inhibitors of digestive enzymes, such as cowpea trypsin inhibitor and wheat α-amylase inhibitor, had no effect, as would have been expected on the basis that sapsucking homopteran insects do not rely on protein or starch digestion for nutrients. On the other hand, two types of protein did show deleterious effects: lectins and oxidative enzymes such as lipoxidase and, to a lesser effect, polyphenol oxidase. The toxicity of lectins varied considerably from those that had very little effect on the corrected mortality at the concentration used (0.1% w/v in the liquid diet), e.g., the lectin from garden pea, to those that gave corrected mortality values of nearly 90%, e.g., the lectins from wheat germ and from snowdrop. The results of many similar bioassays have suggested that BPH is generally sensitive toward insecticidal proteins, so results obtained with this species must be extended with

caution to other insect pests. Nevertheless, assays with GLH showed that the lectins from snowdrop and wheat germ were both strongly toxic toward this species also, although it was not sensitive to lipoxidase.

Toxicity of lectins: The bases for the toxicity of lectins toward animals, in general, are still the subject of research. In higher animals, binding of lectins to gut epithelial cells is well demonstrated, and effects on the growth of gut tissues, particularly in terms of effects on the normal structures of villi, are well documented (Pusztai et al. 1991). Certain lectins also show systemic effects by crossing the gut wall intact and passing into the circulatory system. An additional factor is the effects of lectins on the attachment of gut microflora to the gut epithelium, which can lead to breakdown of the gut wall and bacterial invasion of gut tissues. All these effects are mediated through the carbohydrate-binding properties of lectins, which lead to interactions with cell surface glycoproteins, both on gut epithelial cells and on bacteria. The situation in insects is less clear. Binding of lectins to gut surfaces in insects has been observed by several researchers, but the results of this binding are not characterized. The toxicity of wheat germ lectin toward a range of insects and its specificity of binding toward chitin have led to suggestions that the peritrophic membrane, a thin porous chitin layer that covers the gut epithelium in many insects, is the target of its action. However, other chitin-binding lectins are not toxic, and lectins with other carbohydrate-binding specificities are toxic (Powell et al. 1993, 1995). The toxicity of many lectins toward higher animals limits their usefulness in the protection of crop plants that are intended for consumption. In particular, wheat germ lectin, which is strongly insecticidal, is also significantly toxic to mammals and other higher animals. However, certain lectins, in particular those from the plant family Amaryllidaceae, show low or no toxicity toward higher animals, but are toxic to insects. This type of lectin is exemplified by snowdrop (*Galanthus nivalis*) lectin (GNA), which had been identified as toxic to BPH. GNA was thus selected as the "best candidate" gene for engineering of BPH-resistant rice.

2 Conventional Host-Plant Resistance Strategies

At organismal level of an insect, mainly insect's growth, survival and reproduction is affected by plant secondary metabolites (Anonymous 2014), without varying their constitution, can perform defense against pathogens, herbivores, some abiotic stresses and during competition with other organisms and while showing mutualistic behavior (Ogbemudia and Thompson 2014). All this leads not only to decrease the dependence on synthetic pesticides, besides improvement in the fitness of a particular plant's herbivore species (Mouttet et al. 2013), but also improvement of the ecological environment (An et al. 2014); Herbivorous species generally cannot detoxify or impound specific secondary plant compounds. In this context, the larvae of both specialist (*Manduca sexta*) and generalist (*Spodoptera exigua*) lepidopteran herbivores in its native habitat attack the plant *Nicotiana attenuata*. The specialist species, *Manduca sexta* is highly tolerant to nicotine, which is a metabolite associated with defense mechanism of plants.

The exceptional tolerance of *Manduca sexta* is due to rapid excretion and cytochrome-mediated oxidative detoxification (Kumar et al. 2014). Allelochemicals produce negative effects on specialist herbivores, when very large amounts of these compounds are produced by a new cultivar. The concentrations of tannin present in the roots and shoots of plants is related to *Bikasha collaris*, the insect tallow tree specialist beetle which exhibited all the positive and negative effects when chemical analysis of plants with varying phenolics was done. When caterpillars attack the host plant, salicylic acid and jasmonic acid are produced and reduce the effect of the attack by aphids (Ali and Anurag 2014). Therefore, the function of specific plant volatile products is to either enable or restrict herbivore for oviposition or feeding. (Huang et al. 2014), e.g., strong metabolite effects are shown by rice (*Oryza sativa*) toward insects.

In transgenic plants *Bt* toxins and some other plant metabolites may be directly or indirectly passed onto biological control agents, which at the end affect the natural enemies. The breeding programs, during the changes in plant characteristics, have either positive or negative or neutral effect on insects. The aim of some specific breeding programs is to produce physical plant barriers such as glandular trichomes or an altered layer of wax or plant allelochemicals. The performance of herbivore is negatively affected by these plant barriers, specifically in some nectarless cultivars, since herbivores are attracted by nectar and cotton plants.

Reducer of plant digestibility (antibiosis), which is believed to slow down the development of herbivore characteristics, is an example of positive effect on carnivores. Carnivore effectiveness may be increased due to the stage of herbivore insect that is susceptible to their enemies for longer duration. Ants or mites use some plant structures such as domatia, which provide shelter to them. These can also enhance the effectiveness of carnivores. Conventional host-plant resistance may be seen on carnivores when herbivores seize the activity of producing toxins which are ultimately used by them to defend against bioagents, which are their carnivore enemies, especially in specialist herbivores. Carnivores are generally very much able to deal with herbivores, sequestered plant toxins as compared to generalist carnivores. A number of plant tissues and plant products may be used as nutrition source such as plant sap and pollen and floral and extrafloral nectars. These are the toxins which natural enemies may face either directly or indirectly.

They show specific insecticidal effect on the orders Hymenoptera (bees and wasps), Coleoptera (beetles and weevils) (López-Pazos et al. 2010; Sharma et al. 2010), Diptera (flies and mosquitos), and Lepidoptera (butterflies and moths) (Baig et al. 2010; Darsi et al. 2010) and to non-insect species such as nematodes (Hu et al. 2010). *Bt* toxins are of great importance as the main biological control agent, and as compared to chemical insecticides, they get major preference. The multiple sites of action of *Bt* toxins are the main reason of its efficacy against the insect pests. Its environmentally friendly nature has been shown by various assessments, without any significant adverse effect (Chen et al. 2011; Randhawa et al. 2011).

The nonselective lethal effects of the orders Coleoptera (beetles and weevils), Diptera (flies and mosquitos), Hymenoptera (bees and wasps), and Lepidoptera

(butterflies and moths) and to non-insect species such as nematodes (Stevens et al. 2011) and the fast development of insect pest resistance to synthetic insecticides are the reasons of the increased popularity of *Bt* toxin as a biological control agent over the synthetic chemicals. A major role is played by transgenic crops in protecting the crop against its main insect pest enemies and also in providing a valuable resource for suppressing insect pest (Benjamin et al. 2014). Favourably, scientists were able to construct the resistant or tolerant plants and whether there is reduction in chemical pesticide usage or additional increase in crop yield is achieved; thus, the transgenic crop will be dependent on the efficacy. At the same time, many insect pests are not susceptible to available range of insecticidal crystal protein genes. Very little or no attention from this technology has been received by a number of serious pests of local crops. The majority of genes of these pests cannot be treated or have developed resistance needs to be broaden (Karthikeyan et al. 2012).

The majority of pests are controlled by using transgenic crops worldwide. There is a need for understanding of the factors which affect the responses to natural selections to develop the strategies for delaying the evolution of pests' resistance to *Bt* crops; it also includes variation in survival on *Bt* crops.

3 Emerging Genetic Engineering Approaches in Plants

Expression of proteins in transgenic plants phloem-specific promoters against exposure to insecticides and constitutive CaMV35S gene promoter, used in many constructs for expression in transgenic plants, is expressed efficiently in phloem tissue, it was desirably felt to identify promoters that would show phloem-specific expression for use in producing rice with BPH resistance. Use of such promoters could give higher levels of expression in the phloem and would minimize exposure of non-target insects and other consumers of the plant material to GNA. Use of an endogenous phloem-specific promoter was decided on. Protein concentrations in phloem of different plant species have been estimated at 0.03–0.2% (w/v) in most species or as much as 10% in cucurbits, and thus the lower limits of effectiveness of GNA lie within achievable expression levels. Sucrose synthase is known to be specific to phloem tissue and studies on the gene that encodes the enzyme from maize had suggested that the promoter was active and phloem-specific. A gene, designated RSs1, corresponding to the maize Sh1 locus was isolated from rice and was fully characterized and sequenced (Wang et al. 1992). The promoter sequence from this gene has been fused to the glucuronidase (gus) gene coding sequence in a promoter-reporter gene construct and transformed into tobacco plants by standard techniques.

Histochemical staining of the transformed plants with X-glc has shown that the RSs1 promoter fragment used (approx. 1.2 kb of 5′ flanking sequence, the transcription start sites, the first intron and the translation start sites) is sufficient to direct phloem-specific expression of gus in transgenic tobacco plants. Expression is observed in phloem sieve tubes and companion cells in roots, stems, petioles, and leaves and is not seen in mesophyll cells or other vascular tissues. The

phloem-specific expression directed by this promoter is thus confirmed (Shi et al. 1994). Expression levels observed in tobacco were low due to the presence of the first intron of the RSs1 gene in the 5′ un-translated sequence between the transcription start sites and the translation start sites. An alternative strategy to isolate a phloem-specific promoter was also followed by attempting to isolate the promoter from a gene encoding one of the phloem-specific P-proteins. An advantage of these genes is that their products are not selectively accumulated in developing seeds, unlike sucrose synthase. A protein to cDNA to gene route isolated a gene encoding a P-protein. Relatively large amounts of phloem exudate from *Cucurbita maxima* (pumpkin) plants were collected and used as a source for purification of the chitin-binding phloem lectin protein designated PP2, a major protein in phloem sap. The partially purified protein was run on SDS-PAGE, and the most abundant polypeptide was blotted onto PVDF membrane and subjected to protein sequencing. This polypeptide was found to have a blocked N-terminus, so to obtain useful sequence information, the separated polypeptide was cleaved in the gel slice by CNBr, and the resulting fragments were purified by reversed-phase high-performance liquid chromatography and sequenced. Two fragments were identified. Amino acid sequence data from these polypeptides were used to generate oligonucleotide sequences of lowest redundancy. These were used as probes on a northern blot of RNA isolated from different organs of developing pumpkin seedlings.

For the hybridization, mRNA species of approx. 0.9 kb in RNA from hypocotyls and this tissue was used as a source for cDNA library construction. The library, in the l phage vector ZAPII, was screened with the labeled oligonucleotide, and positive plaques were purified. Three clones were fully sequenced. These proved to contain identical PP2 lectin-encoding sequences. The sequence predicted by these clones was in complete agreement with the 78 residues of amino acid sequence determined for the PP2 protein, confirming their identity. The PP2 cDNA was used as a probe to screen a cucurbit genomic library in the vector l EMBL3 to obtain a gene encoding the PP2 protein. The gene was fully characterized and sequenced. The predicted amino acid sequence in this gene was not identical to that predicted by the cDNA, but encoded a PP2-like protein. PCR of the *C. maxima* seedling cDNA library using primers specific for the gene sequence amplified a fragment of the expected size (data not presented). This result suggests that a cDNA corresponding to the gene is present in the library and that the gene is highly likely to be expressed. However, when the promoter region was fused to a gus reporter gene and the construct was transformed into tobacco plants, no expression of the reporter gene was observed. The reasons for this failure to observe expression are under investigation. The RSs1 gene had provided a viable phloem-specific promoter, which was used in subsequent constructs. The GNA coding sequence was assembled into two constructs for expression in transgenic plants. A standard transcriptional fusion with the CaMV35S promoter was made for expression in model systems in experiments to "prove" the technology, and a translational fusion between the RSs1 promoter and the GNA sequence, which introduced the translational start of sucrose synthase and some "linker" amino acids onto the N-terminus of the GNA precursor, was made for expression in both model systems and rice. The RSs1-GNA construct was

introduced into tobacco via standard *Agrobacterium tumefaciens* transformation procedures. The phloem-specific expression pattern observed with the reporter gene gus driven by the RSs1 promoter was also evident with GNA in the transformed plants. GNA accumulation was determined by immunohistochemical staining (Shi et al. 1994), and the presence of GNA in the phloem vessels and companion cells was demonstrated.

4 Isolation of Different Insect Pest-Resistant Genes from Various Microorganisms

The VIP genes from *Bacillus thuringiensis* and *B. cereus* are the closest to cry genes which are commercially used against insect pests. The expression of *Bt* toxins is restricted to sporulation, while insecticidal proteins of VIP genes are expressed in the vegetative stage of growth which starts at mid-log phase as well as during sporulation. So far more than 50 VIP proteins have been identified. It is found that swelling and disruption of the epithelial cells of the midgut and peritrophic membrane by osmotic lysis in the target insects are caused by the ingestion of VIP proteins (George and Crickmore 2012). Binary toxins are the components of VIP toxins. These binary toxins contain of two components, Vip1 and Vip2, and their combinations are considered highly lethal to insects, e.g., western corn rootworm (*D. virgifera*), but their insecticidal activity is not seen against lepidopteran insects.

The other group consisting of Vip3 toxins and Vip3 Aa1, which is the first identified Vip3 toxin, shows highly insecticidal effects against a number of lepidopteran pests of cotton and maize. Boll weevil (*A. grandis*), whose larvae were killed by a highly effective protein (cholesterol oxidase), was discovered in the culture filtrate of *Streptomyces*. When cholesterol oxidase is ingested, the morphological changes induced suggest that a direct effect on boll weevil's midgut tissue is seen, which at low dose disrupted the epithelium of midgut and at higher doses lysed its cells.

Plants which express cholesterol oxidase choM gene, in transgenic leaf tissue in transformed tobacco (*Nicotiana tabacum* L.) plants, exerted insecticidal activity which includes serious developmental abberations against boll weevil larvae. An observation on bacterium *Photorhabdus luminescens* was done by Bowen et al. The larvae live in the gut of entomophagus nematodes. The bacteria to which an insect is infected with, releases them into the hemocoel of the insect, the insect dies and the bacteria and nematodes start replicating in the remains of the insect. The toxin complex loci tca, tcb, tcc, and tcd encode a series of four native complexes which make the toxin. Both tca and tcd represent strong alternatives to *Bt* for transgenic distribution and encode the complexes containing high toxicity to tobacco hornworm (*Manduca sexta*).

Liu and his colleague have stated that, tcdA gene of *Photorhabdus luminescens* which encodes a 283-kDa protein was introduced into *Arabidopsis thaliana* L. (Liu et al. 2002). The toxin is highly detrimental to a variety of insects, and highly effective against southern corn rootworm and tobacco hornworm, has shown toxin A

expression above 700 ng/mg. In the same way, a number of protein inhibitors in field crops show resistance to agriculturally important pests, which is produced by transgenic expression. The formation of a lytic pore in the epithelial membrane of the midgut is induced by the activated toxins, resulting in lysis of cell, interruption of feeding, and death of larva. Further, retarded insect larval development and antibiosis development are caused by the digestive inhibitor proteins, produced by the transgenic crops. Lethal effects to herbivores were shown by the two types of proteins polyphenol oxidase and lectins and lipoxidase.

5 Recent Development in Rice Functional Genomics Research

In the past decade, a number of functional genomics platforms have been established, which also include germplasm resource collection and mutant libraries generation, full-length cDNA libraries, microarrays of gene expression, and RNA sequencing (RNA-Seq) technologies for profiling of expressions (Jiang et al. 2012; Yang et al. 2013a). Phenomics, metabolomics, and proteomics platforms have gradually been established and improved, and corresponding bioinformatics analysis platforms and databases have also been set up in rice (Rajasundaram and Selbig 2016). Rice has a rich germplasm resource, including naturally found and artificially modified germplasms. There are 21 wild and 2 cultivated species of the genus *Oryza*, which are further subdivided into 10 different genome types (Vaughan et al. 2003).

The International Rice Research Institute (IRRI) has the world's most diverse and largest collection, as it has maintained 110,000 rice germplasm accessions. The genomes of a number of germplasms have been sequenced again along with developed sequencing technologies of the next generation, which help in better understanding of molecular basis of agronomically significant traits (Xu et al. 2011). The resequencing of 3000 rice varieties was performed recently by the Chinese Academy of Agricultural Sciences together with IRRI and Huada Gene Research Institute (China). A total of 18.9 million insertions/deletions (InDels) and single-nucleotide polymorphism were identified through genome of Nipponbare alignment. For genomic breeding, exploration of variations of gene sequence from wild rice is of great importance. The genomes of wild rice have also been explored by some international peers. Launching of the Oryza Map Alignment Project (OMAP) done at the University of Arizona in year 2005 is the best example (Wing et al. 2005). At the same time Ds/dSpm tagging, T-DNA insertion, Tos17 tagging, and chemical/irradiation mutagenesis have generated multiple rice mutant libraries.

For the detection of various tissues in elite hybrid rice Shanyou 63 and its parents Zhenshan 97 and Minghui 63 expressions in different conditions, Affymetrix GeneChip Rice Genome Array technique was applied, which was useful for giving the information regarding the Collection of Rice Expression Profiles (CREP) (Wang et al. 2010). This technique was also utilized by Wang et al. (2014a) for the analysis of the expression quantitative trait loci (eQTLs) in case rice seedlings and flag leave

at the time of heading period from recombinant inbred lines (RILs) derived from a cross between Zhenshan 97 and Minghui 63. A number of cis- and trans-eQTLs were observed which control the expression of genes, triggering the formation of the controlling network via gene co-expression investigation (Wang et al. 2014a, b), whereas the other technique, namely, gene expression microarrays, played a significant role in analyzing and comparing the transcriptomes of super hybrid rice LYP9 and its parental cultivars 93–11 and PA64s (Wei et al. 2009). By the use of laser microdissection and RNA-Seq technology, gene expression profiles of reproductive meristems in early inflorescences at the time of early embryogenesis were studied (Harrop et al. 2016; Itoh et al. 2016).

6 Strategies for Recognizing Insect Pest-Resistant Genes in Rice

Transgenic crops modified by cry (Bt) genes obtained from the bacterium *Bacillus thuringiensis* are the firstly used insecticidal genes for plant transformation. *B. thuringiensis* is a gram-positive bacterium producing highly insecticidal protein crystal toxins during sporulation. The digestive system of the insect is the first target of this toxin. After ingestion by susceptible insects, toxins bind to specific receptors in the gut and are solubilized and activated by proteinases in the insect midgut epithelium. More than 400 genes encoding toxins from a wide range of *B. thuringiensis* mosquitoes have been identified so far (Baig et al. 2010; Darsi et al. 2010). Various assessments have shown that *Bt* is mostly environmentally friendly without significant adverse effects (Chen et al. 2011; Randhawa et al. 2011). The popular biological control agents over synthetic chemicals is because of the nonselective lethal effects of the latter agents (Stevens et al. 2011) and the rapid development of the resistance by insect pests to synthetic insecticides, whereas the transgenic crops play a central role in protecting the crop from its major insect pests and provide a valuable resource for insect pest suppression (Benjamin et al. 2014). Development of strategies to delay the evolution of pests resistance to *Bt* crops requires an understanding of the factors affecting responses to natural selection, which include variation in survival on the Bt crops, heritability of resistance, and fitness advantages associated with resistance mutation.

Genetic engineering as an evolving approach in plants mostly involves the addition and integration of genetic material (single or multiple genes) into a recipient plant, leading to the modification of the plant genome. The plant with modified genome is known as transgenic plant or genetically modified plants (Pandey et al. 2011). Useful traits responsible for resistance against the insect pests have been transferred to crop varieties from noncultivated plants, for decades. Plant improvement whether as a result of natural selection or the efforts of plant breeder has always relied upon evolving, evaluating, and selecting the right combination of alleles. Moreover, the transgenic research has made significant progress not only just widening the genetic pool of useful genes but also permitting the introduction of a number of different desirable genes at a single event. Besides, the introduction of

molecular changes by genetic engineering takes less time compared to conventional genetic methods. Hence, genetic engineering for developing insect pest-tolerant plants based on the introgression of gene might be a faster track toward improving crop varieties, not only in terms of yield parameters but also in offering resistance to insect pests (Karthikeyan et al. 2012).

The Knowledge-based Oryza Molecular Biological Encyclopedia (KOME) database collects information for about 38,000 full-length cDNAs of japonica cv. Nipponbare. The Rice indica cDNA Database (RICD) database contains 10,081 and 12,727 full-length cDNA sequences from Gaungluai 4 and Minghui 63, respectively. Affymetrix GeneChip Rice Genome Array was used for the analyses of specific expression profiles in various tissues under different stress conditions in elite hybrid rice Shanyou 63 and its parents Zhenshan 97 and Minghui 63, which were in the information platform of the Collection of Rice Expression Profiles (CREP) (Wang et al. 2010). Gene expression microarrays were used to analyze and compare the transcriptomes of super hybrid rice LYP9 and its parental cultivars 93–11 and PA64s (Wei et al. 2009).

Gene expression profiles of reproductive meristems in early inflorescences and the spatial and temporal transcription a laser microdissection and RNA-Seq approach (Harrop et al. 2016; Itoh et al. 2016). Wang et al. (2014a) used an Affymetrix GeneChip Rice Genome Array to analyze the expression quantitative trait loci (eQTLs) in rice seedlings and flag leaves during heading period from recombinant inbred lines (RILs) derived from a cross between Zhenshan 97 and Minghui 63.They found a large number of cis- and trans-eQTLs that regulate the expression of genes, leading to the construction of the regulatory network through gene co-expression analysis (Wang et al. 2014b). Further analysis of the flag leaves of an immortalized F2 (IMF2) population identified many genomic loci that control the expression abundance of small RNAs (Wang et al. 2015), providing new insight into the regulation of gene expression.

The construction of high-throughput and accurate phenomics platforms is becoming a new research area of rice functional genomics. CropDesign (Belgium) developed the TraitMill platform, which can be used to measure the traits such as aboveground biomass, plant height, total number of seeds, total number of filled seeds, total weight of seeds, and harvest index, which are useful for breeding applications (Reuzeau et al. 2010). LemnaTec Scanalyzer 3D enabled the fully automatic analysis of plant phenotypes. The Australian Plant Phenomics Facility has successfully applied this technology in the studies of salt stress (Rajendran et al. 2009), drought tolerance, toxicity (boron) tolerance (Schnurbusch et al. 2010), as well as modeling and prediction of crop yield (Golzarian et al. 2011) and root development (Rahnama et al. 2011). In 2011, LemnaTec (Germany) and KeyGene (the Netherlands) announced the commencement of commercial operation of a plant phenomics platform (PhenoFab), which has been officially applied in commercial crop breeding. Huazhong Agriculture University, in collaboration with Huazhong University of Science and Technology, developed a high-throughput rice phenotyping facility (HRPF), which allows the growing of 5472 pots of rice and can automatically monitor 15 important agronomic traits (Yang et al. 2013b, 2014).

Currently, HRPF has been adapted for phenotyping other crops such as rice, maize, rape, and cotton.

Epigenomes play important roles in gene expression reprogramming during cell differentiation, plant development and growth, and response to stress. DNA methylation widely occurs in the genomes of animals and plants, and plays important roles in inhibiting transposon activities, repressing gene expression, and stabilizing the genomes. Compared with *Arabidopsis*, the features of DNA methylation in rice include the following: (1) The average methylation levels of CG, CHG, and CHH and the total cytosine methylation density in rice genome (44.46%, 20.14%, 4.02%, and 24.7%, respectively) are much higher. (2) The methylations of CG and CHG occur mainly in the heterochromatin regions, which modify the transposable elements and related genes. However, CHH methylation is distributed mainly in euchromatic regions and modifies certain transposable elements with shorter lengths, such as miniature inverted-repeat transposable elements (MITEs) (Zemach et al. 2010; Tan et al. 2016). CHH methylation has a profound effect on gene expression in cereal crops (Tan et al., 2016). (3) Methylation in CHG and CHH sequences participates in inhibiting the expression of many functional genes in rice. (4) The methylation level of CG is relatively stable in various tissues during the development process, but the levels of CHG and CHH increase with the development processes (Zemach et al. 2010).

In rice, research on histone modification has been mainly focused on the acetylation and methylation of histone lysine. H3K27ac and H3K9ac share similar distribution patterns in the genome and mainly accumulate in the promoter region. H3K9ac andH3K27ac are highly correlated with gene transcription, suggesting the synergistic effects of the acetylation of different loci on gene expression (Du et al. 2017). H4K16ac and H3K23ac are enriched in genes with low expression and around the transcription start sites (Lu et al. 2015). Currently there are also studies of histone methylation on H3K4, H3K9, H3K27, andH3K36 in the rice genome. The distribution of H3K4me2/3 in the genome is similar to that of H3K9ac and H3K27ac modification and is also located in euchromatin. The genes modified by H3K27me3 show dynamic variations in different tissues from callus, seedling, and shoot apical meristem to inflorescence meristem and play important roles in maintaining the inhibition of genes (Liu et al. 2015a).

The CRISPR/Cas9 system has become a prevalent tool for gene mutagenesis in rice functional genomics research. Feng et al. (2013) used the CRISPR/Cas9 system harboring single-guide RNA driven by OsU6–2 promoter for the disruption of genes ROC5, SPP, and YSA in rice. In the T1 transgenic plants, the mutation rate of SPP was 5%, and that of ROC5 and YSA was as high as 26%–84%. Miao et al. (2013) designed CRISPR/Cas9 system targeting either the CAO1 or the LAZY1 and observed the expected mutant phenotypes. Ma et al. (2015, 2016) developed a robust CRISPR/Cas9 system for high-efficiency multiple genome editing in plants. With this system, an average mutation rate of 85.4% was obtained for the 46 target sites in rice. Currently, the CRISPR/Cas9 system can be used to knock out completely the functions of target genes for rice genetic improvement. It was reported that targeted mutation of indica rice allele Sc-i by CRISPR/Cas9 could improve male fertility in

japonica-indica hybrids (Shen et al. 2017). Recently, significant progress has been made in the generation of targeted point mutations in rice. By using the strategy of homology-directed repair-mediated targeted gene replacement, Sun et al. (2016) introduced Cas9/guide RNA and repair templates simultaneously into rice and obtained multiple discrete point mutations in ALS gene. Li et al. (2016) reported the use of a nicked Cas9 with only the D10A mutation (nCAS9) fused with a cytidine deaminase enzyme and the uracil glycosylase inhibitor to generate point mutations at a specific locus. Lu and Zhu (2017) developed a base-editing system in rice using rat APOBEC1, providing a simple and highly efficient base replacement method for plant molecular breeding.

Huang et al. (2010) identified 3.6 million SNPs by sequencing 517 japonica and indica rice landraces and constructed a high-density haplotype map. GWAS for 14 agronomic traits revealed 37 associated loci, which could explain 36% of the phenotypic variance. GWAS for the flowering time and grain-related traits was conducted with 950 varieties collected worldwide, resulting in the identification of 32 new loci (Huang et al. 2012). Large-scale GWAS on 38 agronomic traits identified 130 associated loci through developing an integrated genomic approach to construct a genome map for 1495 elite hybrid rice varieties and their inbred parental lines, which provided a global view of heterosis from a representative number of hybrid combinations (Huang et al. 2015).

7 Functional Genomics and Genetic Engineering Approaches for the Development of Insect-Resistant Rice

Since the completion of whole-genome sequencing in rice, various functional genomics platforms have been established in the past decade, including collection of germplasm resources and generation of mutant libraries, full-length cDNA libraries, gene expression microarrays, and RNA-sequencing (RNA-Seq) technologies for expression profiling (Jiang et al. 2012; Yang et al. 2013b). Platforms of metabolomics, proteomics, and phenomics have also been gradually established and improved, and corresponding platforms of bioinformatics analysis and databases have also been set up in rice (Rajasundaram and Selbig 2016). Rice (Oryza sativa) has been recognized as a model for plant functional genomics research due to its small genome size, accurate genome sequences characterized by co-linearity with the sequences of other cereal crops, high-efficiency transformation technology, and abundant germplasm resources (Jiang et al. 2012).

Rice is rich in germplasm resources, including naturally occurring and artificially modified germplasms. The genus *Oryza* consists of 21 wild and 2 cultivated species, which are classified into 10 different genome types (Vaughan et al. 2003). The International Rice Research Institute (IRRI) maintained 110 000 rice germplasm accessions, which is the world's largest and most diverse collection (http://irri.org/). To better understand the molecular basis of agronomically important traits, the genomes of many germplasms have been resequenced with the development of next-generation sequencing technologies (Xu et al. 2011). Recently, the Chinese

Academy of Agricultural Sciences together with Huada Gene Research Institute (China) and IRRI performed deep resequencing of 3000 rice varieties (3000 rice genomes project, 2014). Through alignment with the genome of Nipponbare, a total of 18.9 million single-nucleotide polymorphisms (SNPs) and insertions/deletions (InDels) were identified. Exploration of gene sequence variations from wild rice is also of great significance for genomic breeding. Some international peers have also explored the genomes of wild rice. For example, the Oryza Map Alignment Project (OMAP) was launched at the University of Arizona in 2005 (Wing et al. 2005).

Meanwhile, multiple rice mutant libraries generated by T-DNA insertion, Ds/dSpm tagging, Tos17 tagging, and chemical/irradiation mutagenesis have been developed. A total of 246,566 flanking sequence tags from rice mutant libraries with T-DNA, Ds/dSpm, or Tos17 insertion were obtained, targeting 211,470 unique sites (Yang et al. 2013b). Currently, 57% of non-transposable element-related genes in rice have insertion tags (Jiang et al. 2012). Due to the nonrandom distribution of T-DNA and transposon insertions in the genome, it is almost impossible to make every coding gene contain at least one insertion tag. To generate mutations at the whole-genome level, fast neutron irradiation has been used to generate a mutant library in rice variety Kitaake (Li et al. 2016, 2017). Genome-wide sequencing of 41 mutation lines revealed that 1284 genes were mutated and single base substitution was the most abundant mutant type (Li et al. 2016, 2017). Recently, a genome-wide mutant library has been generated using CRISPR/Cas9 (Lu and Zhu 2017; Meng et al. 2017). Collectively, these mutant resources are of great value for both functional genomics and genetic improvement in rice.

Epigenetic modifying factors regulate gene transcription through altering the chromatin states, affecting the growth and development and adaptation to the environments. For example, the H3K27 methyltransferase gene SDG711 and the H3K4 demethylasegene gene (JMJ703) have agonistic functions in reprogramming the H3K27me3/H3K4me3 ratio and modulating gene expression in the inflorescence meristem (Liu et al. 2015b). Epigenetic modifications can also affect the expression patterns of genes in the hybrid progeny. Guo et al. (2015) found that the specific modification sites of H3K36me3 can enhance the expression of some specific alleles in F1 hybrids. A recent study showed that histone deacetylase OsSRT1 directly inhibits the metabolic pathways of glycolysis through mediating the deacetylation of the key enzyme in histone degradation and glycolysis and affects starch accumulation and transposon repression to regulate normal seed development in rice (Fang et al. 2016).

Proteomics studies in rice have been performed mostly using gel-based (1DE, 2DE, and 2DIGE) and gel-free (LC-MS/MS or MudPIT) approaches and more recently iTRAQ (isobaric tags for relative and absolute quantitation) for protein quantitation based on MS/MS. Kim et al. (2014) reviewed and summarized the progress in rice proteomics studies from 2010 to 2013, with major focus on rice under diverse abiotic and biotic stress conditions. More recently, an iTRAQ-labeling-based quantitative proteomics strategy was used to investigate the proteomes under high temperature in different rice cultivars. The results showed that high temperature stress induced small heat shock proteins, expansins, and lipid

transfer proteins in high temperature-resistant cultivars (Mu et al. 2017). Polyethylene glycol-simulated drought responsiveness in a time-dependent manner in root demonstrated that most of the differentially expressed proteins appeared to be involved in bioenergy and metabolism (Agrawal et al. 2016). By using polypeptides enriched and phosphorized by IMAC, 201 phosphopeptides showing pistil-specific expression were identified (Wang et al. 2014a). Protein phosphorylation is one of the most common post-translational modifications. It was speculated that the regulation of protein phosphorylation plays an important role in the growth and development of plants (Cabrillac et al. 2001). There are more than 1400 genes that encode protein kinases and 300 genes that encode phosphatases in rice (Dardick et al. 2007).

Plants can generate as many as 0.2–one million metabolites (Dixon and Strack 2003). In recent years, with the development of metabolomics analytical technologies, particularly the advance in metabolic profiling based on mass spectra and magnetic resonance imaging, the research fields of metabolomics have been continuously expanded (Saito et al. 2013). Progress has been made in the application of plant metabolomics to the identification of functional genes, dissection of metabolic pathways, and genetic analysis of natural variations through integration with other omics technologies (Kumar et al. 2017). Traditional liquid chromatography-mass spectrometry (LC-MS) includes targeted and untargeted metabolomics. A platform of metabolomics based on broad-spectrum untargeted metabolomics analysis has been established, which can quantify more than 800 known and unknown metabolites within 30 min (Chen et al. 2014). The metabolomic analysis of samples from 210 RILs derived from a cross between two elite indica rice varieties, Zhenshan 97 and Minghui 63, and detected approximately 1000 metabolites, which were resolved to over 2800 metabolic QTLs (Gong et al. 2013). Genome-wide association study (GWAS) was employed to identify several hundreds of loci/sites that control natural variations in metabolite contents (Chen et al. 2014), and they annotated over 160 new metabolites, including flavonoids, vitamins, and terpenes.

Rice functional genomics research is aimed at exploring the genes and molecular regulatory networks of agronomically important traits and applying them in varietal improvement, for traits like yield, quality, disease and pest resistance, nutrient use efficiency (NUE), abiotic stress resistance, and reproductive development. At present, a total of 2294 rice functional genes have been identified. Over 100 QTLs for rice blast resistance have been mapped, distributed on 11 of the chromosomes except for chromosome 3 and densely on chromosomes 6, 11, and 12. Twenty-seven genes have been cloned: Pib, Pi-ta, Pi9, Pi2, Piz-t, Pi-d2, Pi33,Pii, Pi36, Pi37, Pikm, Pit, Pi5, Pid3, Pid3-A4, Pi54, Pish, Pik, Pik-p, Pi-CO39, Pi25, Pi1, Pb1, Pi64, LABR_64-1, LABR_64-2, and Pigm (Deng et al. 2017). Most of the disease resistance genes (R genes) belong to NBS-LRR (nucleotide-binding site and leucine-rich repeat) family. Pigm was recently found to be a gene cluster consisting of multiple NBS-LRR genes, and there are two functional proteins, namely, PigmR and PigmS. PigmR confers broad-spectrum resistance, whereas PigmS competitively attenuates PigmR homodimerization to suppress the resistance (Deng et al. 2017). A single-nucleotide change in the promoter of the bsr-d1 gene confers broad-spectrum blast resistance (Li et al. 2017). To date, at least 40 QTLs for

bacterial blight resistance have been reported in rice. Eleven genes have been cloned, including seven dominant genes (Xa1, Xa3/Xa26, Xa4, Xa10, Xa21, Xa23, andXa27) and four recessive genes (xa5, xa13, xa25, and xa41) (Zhang and Wang 2013).

The major insect pests of rice include planthoppers, leafhoppers, striped stem borer, yellow stem borer, leaf folders, and gall midge. A number of insect-resistant germplasms have been found from both cultivated and wild rice species. Great progress has been made in identifying resistance genes to brown planthopper (BPH), with 31 genes genetically mapped. Among these genes, Bph3 (Bph17), Bph14, Bph26 (bph2), and bph29 have been cloned through map-based cloning. Bph14 is the first cloned gene conferring resistance to BPH (Du et al. 2009; Hu et al. 2017).

8 Conclusion

Functional genomic understanding of an agronomic trait refers to characterization of the genes (including non-coding sequences) and their regulatory networks, which collectively determine the formation and development of the trait. The formation of any trait involves a large array of genes, and the majority of the genes that participate in many processes thus affect the development of many traits (or pleiotropic effects). Data and literature accumulated to date have already clearly depicted such a "net-like" structure between genes and traits. In addition, formation and development of traits are greatly influenced by environmental conditions and also to some extent by field management practices. For functional genomic understanding of agronomic traits, a complex trait such as yield may be divided into subtraits, which in turn are subdivided into components and biological processes, which may be specified by pathways. Genes and regulatory networks then would be characterized for each component trait and process. The ultimate goal of rice functional genomics research is for breeding application. The knowledge, genes, germplasms, and genomic data obtained presently are already sufficient to lead a revolutionary change in strategies and technologies in rice breeding, which can be termed "designed genomic breeding."

The advances in genetic engineering have emerged a powerful modality against the important insect pests. In this context, the viable approach for insect pest control in plants without use of chemicals, it is necessary to negotiate exchange of this transgenic technology at easy terms. Further its integration with the conventional approach for resistance breeding will ensure evergreen revolution crucial for global food security. Therefore, aim to discuss the role of genetic engineering in crop protection.

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Genetic Engineering of Rice for Resistance to Insect Pests

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Abstract

For human being, rice is an important crop both as a source of food and income. As the world population is expected to increase to 9.2 billion by 2050, there is a dare need to increase global food production to overcome the demand of world population. But unfortunately, there are a number of abiotic and biotic factors affecting rice production. Among these factors insect pests is a major hurdle for achieving higher rice production. Annually, an average of 37% rice production losses due to insect's pests and diseases has been observed. Globally, there are around 100 insects species considered as rice pests. Rice plant remains vulnerable to these insects from sowing till harvest. Integrated Pest Management (IPM) including: chemical insecticides to biological control methods. Pesticides in the form of fungicides, herbicides and insecticides are contributing a huge part in rice production and around USD5.37 million are spent on them annually. The best choice and an important tool for the farmers is the use of chemical pesticides due to its high effectiveness, quick mode of action and ease of application. But, unnecessary and excessive use of pesticides leads to environment contamination, decline in beneficial insects and secondary pest outbreak. Moreover, the insects have the ability to evolve themselves against these insecticidal chemistries.

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In current scenario for the safe and sustainable agriculture, there is a need of more efficient, environment friendly, accurate and targeted approach is required. Biotechnology and genetic engineering can provide a hope and safe alternative to the pesticides in rice production. Advances in the said fields have been integrated in classical breading approach. Single gene transformation or gene pyramiding has been used for accumulation of multiple resistant gene in crop variety resistant to pests. Number of transgenic rice varieties have been developed, which are capable to sustain abiotic and biotic stresses and has improved nutritional values. There are number of techniques used to produce insect resistant rice crop varieties including: mutagenesis, introduction of foreign gene (single gene and gene pyramiding), genetically engineered/modified Bt Toxins, transplastomic approaches, oligonucleotide-directed mutagenesis, engineered nucleases, antisense technologies and engineered plant membrane transporters. Although at global, number of insect resistance rice varieties have been developed, till date no such variety of rice has been approved by any country.

Keywords

Rice · Insect pests · Genetic engineering · Bt rice

1 Introduction

Rice is an important crop of Asia, being used as a source of food and economy (Westwood 2008). It is consumed by about half of the world population as a food (IRRI 1989). Over 91% of the rice is grown and consumed in Asia Pacific only; thus it play a vital part in the economy development of this part of the world. Over 90% of the world's rice is produced and consumed in the Asia-Pacific Region (Papademetriou 2000), including Pakistan, China, India, Japan, Korea, Southeast Asia and other adjacent areas with total production of 118.2 million tons (USDA 2015). As world population is expected to increase to 9.2 billion by 2050, there is a dire need to increase global food production to overcome the demand of world population. Therefore, future food production should be aligned with less use of land, water, energy, fertilizers and pesticides as compared to current usage (Popp et al. 2013). Moreover, to enhance the food production, high-yielding varieties of crop should be introduced with improved agricultural techniques including soil preparation, water management and fertilizer usage (Oerke and Dehne 2004).

2 Factors Affecting Rice Production

Rice production and economy are threatened by several biotic and abiotic stress factors. Biotic factors include insect pests, rats, nematodes, snails, birds and crop diseases caused by fungi, bacteria and viruses, while abiotic factors include drought, floods, cold weather, soil salinity, submergence and oxidative stress which are also

responsible for valuable economic losses (Ansari et al. 2015). Pest infestation and crop diseases are the main factors responsible for reduction in rice production. Insect infestation increases with an increase in irrigated rice production, eventually leading to more insecticide usage. Major disease like tungro and yellow dwarf are transmitted by insects. These diseases are responsible for valuable rice production losses (Heinrichs et al. 1985).

Pests are the major hurdle for achieving higher rice production. They invade the rice crops or sometimes live in competitive environment with rice plants for nutrients, resulting in yield decrease. Arthropods are responsible for an estimated 18–20% of the annual crop production losses globally, with significantly a higher impact in the developing tropics of Asia and Africa (Sharma et al. 2017). Annually an average of 37% of rice production losses due to insect pests and diseases has been observed. Globally, there are 800 insect species attacking and damaging all parts of rice plant (Dale 1994). However, around 100 species are considered as pests (Pathak 1970). Among them more than 20 insects including black bug, zigzag leafhopper, rice skipper, rice thrips, rice whorl maggot, mealy bug, mole cricket, ant, armyworm, green semilooper, greenhorned caterpillar, rice bug, planthopper, field cricket, cutworm, green leafhopper, rice caseworm, grasshopper (short-horned) and locust, rice gall midge, rice hispa, stem borer, root grub, root aphid and rice leaf folder cause severe damages and transmit diseases to the rice plant (Pathak and Khan 1994). However, the most destructive insects for rice are the lepidopteran stem borers and the rice leaf folder, causing 10 million tons of decrease in annual rice production. Sometimes, the epidemics of these pests can decrease productivity from 60 to 95% of the crop production (Yambao et al. 1993; Pathak and Khan 1994). Rice plant remains vulnerable to these insects from sowing till harvest (Table 1). The damages appear in the form of chewing, boring and sucking different plant tissues, resulting in distressed plant physiology and reduced crop yield (Nasiruddin and Roy 2012).

3 Role of Pesticide in Rice Production

For the sustainability of conventional agriculture, there is a need to encourage integrated pest management (IPM) at a global level. IPM consists of methodologies that vary from careful and targeted use of chemical insecticides to biological control methods including the use of natural parasites and predators (Sorby et al. 2003). As in the last 40 years, the agricultural production has been increased to double due to the proper management of crop by controlling the pests and diseases with the help of pesticide application. Pesticides are contributing a huge part in agricultural development and global food demand (Wang and Li 2007). Global crop harvest has been increased from 42% in 1965 to 70% by 1990. Without pesticides, 70% of global food production could have been lost to pests (Oerke 2006). The global market size for crop protection compounds was 50.62 billion US dollars in 2017. However, around 5.37 million US dollars are spent on rice protection compounds during 2016 (Grand View Research 2018). Thus, to control the damage, biological and chemical methods are used by farmers. Always, the best choice and an important tool for the farmers is

Table 1 Insect pests of rice and stages of rice plant attached by the pests

	Insect pest	Order	Family
Vegetative stage	Seedling maggots	Diptera	Muscidae
	Rice whorl maggots		Ephydridae
	Rice gall midge		Cecidomyiidae
	Stalked-eyed flies		Diopsidae
	Rice caseworms	Lepidoptera	Pyralidae
	Rice green semiloopers		Noctuidae
	Armyworms and cutworms		Noctuidae
	Rice leaf folders		Pyralidae
	Rice stem borers		Pyralidae and Noctuidae
	Rice leaf beetles	Coleoptera	Chrysomelidae
	Rice hispa		Chrysomelidae
	Grasshoppers	Orthoptera	Acrididae
	Katydids		Gryllidae
	Field crickets		Tettigoniidae
	Mealybugs	Homoptera	Pseudococcidae
	Black bugs	Hemiptera	Pentatomidae
	Rice thrips	Thysanoptera	Thripidae
Reproductive stage	Greenhorned caterpillars	Lepidoptera	Satyridae
	Rice skippers		Hesperiidae
	Planthoppers	Homoptera	Delphacidae
	Leafhoppers		Cicadellidae
Ripening stage	White grubs	Coleoptera	Scarabaeidae
	Root weevils		Curculionidae
	Wire worms		Elateridae
	Ripening seed bugs	Hemiptera	Alydidae
	Stink bugs		Pentatomidae
	Field crickets	Orthoptera	Gryllotalpidae
	Mole crickets		Gryllotalpidae
	Root-feeding mealybugs	Homoptera	Pseudococcidae
	Root aphids		Aphididae
	Soil-inhabiting pests ants	Hymenoptera	Formicidae
	Termites	Isoptera	Termitidae and Rhinotermitidae

Extracted from the book *Insect Pests of Rice* by Pathak M.D. Khan, Z.R. (1994). International Rice Research Institute, the Philippines

the use of chemical pesticides because of pesticides' high effectiveness, quick mode of action and ease of application. Moreover, careful application of pesticides plays vital role in plant protection. In the South East Asian countries rice production was increased by 40 and 30% from 2000 to 2013. This significant increase was possible due to the high numbers of application of pesticides (Gianessi 2014).

4 Conventional Methods of Rice Pest Control

Integrated pest management is considered to be a key player in the rice agriculture sector since the 1950s and has been acknowledged worldwide. But due to the globalization of the planet, intra- and intercontinental movement of the invasive species has been increased (Perrings et al. 2000; Clercq et al. 2011; Walsh et al. 2018). Therefore, proper precautionary measures are mandatory to stop the invasion of pests on crops. The integrated pest management (IPM) is the best approach to curtail the pest attack. Many countries across the globe have adopted IPM practices for rice production based on their socioeconomic status and biogeographic characteristics. These practices include cultural, genetic, mechanical, chemical and biological methods. However, the chemical method such as the use of pesticides keeps the level of pest below economic injury level with the help of regular pest scouting of rice fields.

A variety of pesticides are used in rice production including fungicides, herbicides and insecticides. As per the Royal Society of Chemistry report, there are about 800 different insecticidal chemistries registered worldwide for crop protection. Fungicides are used against sheath blight and blast fungus. These infectious agents are responsible for valuable rice production losses in the past (Supaad et al. 1980; Sreenivasaprasad 2004). A number of fungicides have been used effectively with two spray programmes. Increase in rice production and significant reduction in blast fungus and sheath blight have been observed (Upmanyu and Rana 2012; Mandal and Jha 2008; Kumar 2011).

Weeds compete with rice crop for space and soil nutrients. The conventional technique for weed control in rice field is hand weeding, but this approach is laborious (Rashid et al. 2012). Significant rice crop losses have been observed in Asia due to the negligence of the farmers or the constraint of the labour cost for hand weeding (Savary et al. 2000; Ghosh et al. 2004). In this scenario the use of herbicides is the best choice to avoid the laborious work in the form of hand weeding. Several studies have shown that farmers using herbicides instead of hiring labour results in increase in production and reduction in total expenditure cost (Rashid et al. 2012; Ahmed et al. 2001; Ghosh et al. 2004).

In the 1960s the Green Revolution in Asian agriculture introduced the use of organochlorine pesticide for improved agricultural practices. With the passage of time, more improved neurotoxic insecticidal chemistries were introduced in the form of organophosphates, methyl carbamates and pyrethroids. Organochlorine was introduced into the market in 1942 as a pest control agent. Organochlorine is an acute neurotoxin with three major groups including DDT, hexachlorocyclohexane, and cyclodiene based on active ingredients (Thacker 2002). Dichlorodiphenyltrichloroethane (DDT) attaches itself to the voltage-gated sodium channels of the axon membrane, resulting in abnormal flow of sodium ions across the membrane, eventually, leading to paralysis or death of insect pest, while hexachlorocyclohexane and cyclodiene disrupt calcium ion movement by acting on gamma-aminobutyric acid (GABA) receptors of neuron cell (Roberts et al. 2004; Thacker 2002). Other organochlorine active ingredients are heptachlor, dicofol,

chlordane, endosulfan, aldrin, dieldrin, mirex, endrin and pentachlorophenol are used as pest control agents. However, DDT, chlordane and endrin have been banned due to their off-target toxic effect on wildlife and their long term persistent in environment (Metcalf 2000). Organophosphates are another class of modern synthetic pest control agent of rice. Organophosphates affect the nervous system of insects by irreversibly inhibiting the acetylcholinesterase (AChE), resulting in hyperexcitation of the central nervous system (CNS) due to the accumulation of acetylcholine (Gupta and Milatovic 2012). Organophosphate pesticides are classified based on their hydrocarbon structures, including aliphatic compounds (malathion, dichlorvos, acephate, dimethoate, disulfoton, phorate, methamidophos and mevinphos), phenyl compounds (parathion, fenitrothion and fenthion) and heterocyclic compounds (diazinon, azinphos-methyl and chlorpyrifos) (Thacker 2002; Ware and Whitacre 2004). Carbamate insecticides are the third class of chemical insecticides. They are toxic to mammals and insects. Their mode of action and intoxications are analogous to organophosphate insecticides (Gupta and Milatovic 2012). The active ingredients of carbamates can be divided into three groups, carbocyclic, heterocyclic and aliphatic (Thacker 2002; Ware and Whitacre 2004). The last class of synthetic insecticide used for pest control in rice field is pyrethroids. They are contact and stomach poisons. Pyrethroids disrupt the sodium channel; thus, its insecticidal activity is similar to that of DDT. On the basis of sodium current modification and alcohol moiety, pyrethroids can be classified as type 1 or type 2. The type 1 group compounds are pyrethrins, allethrin and tetramethrin, while type 2 pyrethroids are fenvalerate and fluvalinate. Pyrethroid insecticides are effective against a range of insect pests. Susceptibility to pyrethroid toxicoses is higher in insects than mammals primarily because of slower metabolic clearance, low body temperature and higher affinity for the pyrethroids in insects (Ensley 2007).

An alternative to pesticides usage is the use of nonchemical techniques for insect pest and disease management. It includes the use of disease-resistant varieties against rice blast and planthoppers. Another technique is to change the timing of rice seeding and transplanting. Moreover, duck, fish and frog rearing in rice field is also helpful in controlling planthoppers, rice sheath blight and weeds. Soaked rice seeds with biogas fermentative liquid and use of pest killing lamp are also used for pest control (Sorby et al. 2003; Huang et al. 2014).

Biological control is another approach of integrated pest management (IPM). This approach is environment friendly and lacks the use of hazardous insecticides. It involves the utilization of natural living organism known as enemy to the insect pests. This approach has been successfully used in crop pest management (Mahr et al. 2001). Brown planthopper is one of the most destructive pests of rice controlled by natural enemies used as a biocontrol agent. Biological control includes the use of spiders and coccinellid beetles (Fahad et al. 2015).

5 Adverse Effects of Conventional Methods of Pest Control

An effective integrated pest management (IPM) is very important to increase crop yield. The pest control approaches include chemical, biological, mechanical and nonchemical and use of pest- and disease-resistant varieties. To meet the global food demand and more food production resulted in 15–20-fold increase in pesticides application worldwide. As pesticides play a vital role in food production, they are also responsible for environmental pollution and persistent risk for human health (Oerke 2006). Moreover, lack of proper guidance of the farmers results in unnecessary and excessive use of fertilizers and pesticides, leading to environment contamination, decline in beneficial insects and secondary pest outbreak. Moreover, the use of insecticides in early rice cropping season resulted in the decline of plant beneficial insects, such as spiders and beetles, thus creating a suitable environment for brown planthopper propagation. Furthermore, the insects have the ability to evolve itself against these insecticidal chemistries (Ffrench-Constant 2007; Heinrichs 1994; Fahad et al. 2015). Insects evolve through different resistance mechanisms against insecticides. These mechanisms include behavioural resistance (to avoid exposure to insecticides), penetration resistance (reduced cuticle penetration), target site resistance (mutations in the insecticide target site, e.g. acetylcholinesterase, sodium channel and GABA receptor) and metabolic resistance (increase in the level of detoxification enzymes, e.g. glutathione S-transferase, carboxylesterase, cytochrome P450 monooxygenase, etc.). Insects develop insectcide resistance by evolving single mechanism or combination of these resistance mechanisms (Ffrench-Constant 2007; Hemingway et al. 2004; Li et al. 2007). Similarly, the biological control will not be effective against all pests, but it is useful, inexpensive and secure. Moreover, the biological control methods depend on its relationship with target insect pests and the environment, thus making it more complex as compared to the traditional chemical approach (Sorby et al. 2003; Fahad et al. 2015).

Significant level of pesticide residues has been observed in wheat and rice grains produced from conventional methods of agriculture using pesticides (Rekha et al. 2006). A number of insecticide resistance have been observed in crops fields of cotton, tobacco and vegetables (Zettler and Cuperusi 1990). Insecticide resistance in rice brown planthopper against imidacloprid has been documented (Gorman et al. 2008). The number of resistant insect pests is increasing day by day with an extensive and improper usage of insecticides. Other insect pests including cotton bollworm, whitefly, tobacco caterpillar, cotton aphid, potato aphid, diamondback moth and mustard aphid have developed resistance against insecticides (Sharma et al. 2001; Rasool et al. 2014; Li et al. 2007).

6 Use of Biotechnological Approach in Rice Crops for Insect Pest Control

In the evolving insecticide resistance scenario for safe and sustainable agriculture, there is a need for more efficient, environment-friendly, accurate and targeted approach. Biotechnology and genetic engineering can provide hope and is a safe alternative to the pesticides. Advances in biotechnology and molecular biology have been integrated in classical breeding approach. Molecular markers can be used for germplasm improvement. Similarly, gene pyramiding has been used for accumulation of multiple resistant gene in crop variety resistant to pests and disease. Allelic frequency and DNA barcoding can be utilized to study diversity and desired traits. Further advancements in biotechnology in the form of next-generation sequencing (NGS) and associated technology including RNA sequencing and chromatin immunoprecipitation sequencing are increasing our molecular understanding of important plant breeding traits. Functional gene analysis of the rice traits and genes has been made possible by reverse genetics and advanced mutagenic techniques (Hirochika et al. 2004; Biswal et al. 2017). Bioinformatics tools and searchable database of DNA, RNA, protein and metabolomics are also providing great assistance in understating rice plant (Collard and Mackill 2008). A number of plant tissue culturing techniques have been adopted for crop improvement for the last three decades. Moreover, in vitro fertilization is helpful in overcoming the physiological incompatibility barriers in both interspecific and intergeneric crosses. Protoplast fusion is utilized for producing hybrid plant, which is normally not possible by sexual methods (International Rice Research Institute 2013). A number of transgenic rice varieties have been developed, which are capable to sustain abiotic and biotic stresses and have improved nutritional values (Endo et al. 2007). Lastly, the recent advances such as marker-free transgenic, enzyme system like transcription activator-like effector nuclease (TALENs) and CRISPR/Cas9 can be helpful in crop improvement strategies.

Based on the above mentioned advancements, biotechnology is providing an alternative to chemical control of pest and diseases, as biotechnological control methods have shown encouraging results in crops. One of the main techniques in this approach is the exploration of the insecticidal properties of genes, followed by isolation and insertion of that desired gene to crops. The transgenic plant confers resistance to insect pests. Since the first genetically modified plant herbicide-resistant tobacco in 1983 in France, many transgenic crop varieties have been developed and commercialized, which confer resistance to insect attack. Genetically engineered crops are ensuring sustainability of agriculture by providing more and healthy food (Martineau 2001). Biotechnology plays a significant contribution to global food security, climate change and sustainable agriculture. On the other hand, biotechnological crops have reduced the use of pesticides. In 2016, 670 million kilogram reduction in pesticides usage has been observed. Moreover, it also results in reduced CO_2 emissions by 27.1 billion kilograms.

In the last two decades, the genetically modified (GM) plant cultivation expands rapidly from 1.7 million hectares in 1997 to 2.5 billion hectares in 2018. This is significantly important that among the 26 GM crop-cultivating countries, the majority of them are developing nations (James 2016). As per ISAAA, GM Approval Database in 2018, 26 countries have cultivated 32 approved GM plant species; however, commercial use was observed in 44 countries (Table 2). These GM crops have the traits of insect resistance, herbicide resistance, drought tolerance and virus resistance and stacked traits (containing more than one desired genes). In

Table 2 List of genetically modified plant and crop species registered on the International Service for the Acquisition of Agri-biotech Applications (ISAAA) website till 2019

S.No.	Name of crop and plant	Scientific name	Number of events/varieties
1	Alfalfa	*Medicago sativa*	05
2	Apple	*Malus x domestica*	03
3	Argentine canola	*Brassica napus*	42
4	Bean	*Phaseolus vulgaris*	01
5	Carnation	*Dianthus caryophyllus*	19
6	Chicory	*Cichorium intybus*	03
7	Cotton	*Gossypium hirsutum*	66
8	Cowpea	*Vigna unguiculata*	01
9	Creeping bentgrass	*Agrostis stolonifera*	01
10	Eggplant	*Solanum melongena*	01
11	Eucalyptus	*Eucalyptus* sp.	01
12	Flax	*Linum usitatissimum*	01
13	Maize	*Zea mays*	238
14	Melon	*Cucumis melo*	02
15	Papaya	*Carica papaya*	04
16	Petunia	*Petunia hybrida*	01
17	Pineapple	*Ananas comosus*	01
18	Plum	*Prunus domestica*	01
19	Polish canola	*Brassica rapa*	04
20	Poplar	*Populus* sp.	02
21	Potato	*Solanum tuberosum*	49
22	Rice	*Oryza sativa*	08
23	Rose	*Rosa hybrida*	02
24	Safflower	*Carthamus tinctorius*	02
25	Soybean	*Glycine max*	41
26	Squash	*Cucurbita pepo*	02
27	Sugar beet	*Beta vulgaris*	03
28	Sugarcane	*Saccharum* sp.	06
29	Sweet pepper	*Capsicum annuum*	01
30	Tobacco	*Nicotiana tabacum*	02
31	Tomato	*Lycopersicon esculentum*	11
32	Wheat	*Triticum aestivum*	01

the early era of transgenic crop commercialization, Bt maize and Bt cotton were approved. Both of the transgenic crops express insecticidal protein due to the presence of transgene isolated from bacterium *Bacillus thuringiensis*. These varieties are easily accepted by the farmer community due to decrease in number of insecticide spray and high yield (Shelton et al. 2002).

6.1 Bt Crops Cultivated in Fields Worldwide

There are a number of biological control methods used for insect pests control including the use of parasitic wasp to control aphids, nematodes to kill wine weevil, nuclear polyhedrosis virus (NPV) to kill codling moths, entomopathogenic fungi to kills lepidopteran and *Bacillus thuringiensis* bacteria to limit insect pests of Lepidoptera, Diptera and Coleoptera (Thacker 2002; Moscardi 1999; Faria and Wraight 2007; Shah and Pell 2003; Gill et al. 1992; Höfte and Whiteley 1989; Schnepf et al. 1998). However, for the last two decades, one of the most accepted and adopted pest management approaches is use of genetically engineered crops Bt crops. *Bacillus thuringiensis* express insecticidal δ-endotoxins known as Cry protein. Upon ingestion of Cry toxin by insect, it gets activated in the insect gut and destroys the epithelial cell lining of the gut due to induced osmotic lysis (Bates et al. 2005; Bravo et al. 2007).

Bt genes from *B. thuringiensis* have been isolated and transferred to different crops. Bt crops are genetically modified using biotechnological techniques. These crops express insecticidal protein known as Bt toxin. One of the most commonly grown crops of Bt is sweet corn. The use of this crop is effective against sweet corn pest *Helicoverpa zea* (Shelton et al. 2013). Similarly, adaptation of Bt cotton containing Bt toxin gene is highly effective against lepidopteran polyphagous insect pest, e.g. *Helicoverpa armigera*. Additionally, the cultivation of these Bt crops boosts the population of biological control agent (natural enemies) due to no or less usage of insecticide application (Naranjo 2011). Bt toxin is used against beetles, mosquitoes, black flies, caterpillars and moths.

6.1.1 *Bacillus thuringiensis* (Bt)

Bacillus thuringiensis is a soil dwelling Gram-positive bacteria. It was observed for the first time by Japanese scientist Shigetane Ishiwatari in 1901 as *Bacillus sotto*, a silkworm killer bacteria, (Milner 1994) and isolated by Ernst Berliner from dead Mediterranean flour moth in 1911 in Thuringia state of Germany. *B. thuringiensis* is spore-forming bacterium that produces parasporal crystalline inclusion bodies known as Cry (crystal) and Cyt (catalytic) proteins. The targets of these toxic proteins are insects (lepidopterans, coleopterans, hemipterans and dipterans), nematodes, mollusc (snails) and human cancer cells (Höfte and Whiteley 1989; Schnepf et al. 1998; Van Frankenhuyzen 2009; Ben-Dov 2014; De Maagd et al. 2003; Chougule and Bonning 2012; Van Frankenhuyzen 2013; Ali et al. 2010; Bravo and Soberón 2008). These proteins can be grouped into 73 different types ranging from Cry1 to Cry73 based on protein identity, i.e. 90%. These groups are

Table 3 representative insecticidal proteins from *Bacillus thuringiensis*

Bt insecticidal protein	Target insect order
CyIAa	Lepidoptera
CryIAb	Lepidoptera
CryIAc	Lepidoptera
CryIBa	Lepidoptera
CryICa	Lepidoptera
CryIDa	Lepidoptera
Cry2Aa	Lepidoptera and Diptera
Cry2Ab	Lepidoptera
Cry3Aa	Coleoptera
Cry3Ba	Coleoptera
Cry4Aa	Diptera
Cry4Ba	Diptera
Cry10Aa	Diptera
Cry11Aa	Diptera
Cry11Ba	Diptera
CytIAa	Diptera
Cyt2Aa	Diptera

Extracted from research paper "Genetically engineered (modified) crops (*Bacillus thuringiensis* crops) and the world controversy on their safety" by Abbas, M.S.T. (2018). *Egyptian Journal of Biological Pest Control*

further classified into subgroups by adding uppercase letters A, B, C, etc. based on 95% identity. Further, minor variations in these groups are represented by lowercase letters a, b, c, etc., for example, Cry1Ac, Cry2Ab, etc. Among them Cry1 and Cry2 proteins are used against lepidopteran insects. However, Cry3 protein is effective against coleopterans and Cry4, Cry10 and Cry11 against dipterans. These insecticidal proteins are effective against the larval stage of insect (Table 3).

The Cry insecticidal protein is produced in crystal form. As these Cry proteins are species specific, they have narrow range of target and are harmless to natural enemies of insects. Moreover, biosafety studies have proven these insecticidal proteins have no effect on human beings and other vertebrates (Höfte and Whiteley 1989). Some stains of *B. thuringiensis* produce Cyt toxic protein crystals. These toxins are active against a wide range of invertebrates and vertebrates. Moreover, the presence of Cyt protein enhances the insecticidal efficiency of Bt toxic protein against dipteran and some coleopteran insects.

6.1.2 Mode of Action of Bt Insecticidal Protein Against Targeted Insects

The insect's larvae attack on Cry toxic protein expressing crops. The larvae chew the vegetative part of crops plant, e.g. plant leaf. The ingested plant materials pass to the larvae gut, where the Cry toxin is cleaved by proteolytic enzymes of the gut in the alkaline pH and is converted from pro-toxin form to toxin form (Bravo et al. 2007). The activated toxin interacts with receptors of the apical microvillar brush border

membrane of the epithelial cells of the midgut. This interaction results in swelling and lysis of these epithelial cells. The gut juices make its way to the hemocoel, resulting in an increase of pH of haemolymph, which ultimately leads to insect paralysis and death (Bravo et al. 2007; Soberon et al. 2010). Natural enemies of the insect pest and other organisms including human and vertebrate animals do not have Bt insecticidal toxin bind sites (receptors). Thus, these toxins cannot cause toxicity in said organisms (Gill et al. 1992). The mode of action of the Cyt insecticidal protein is similar to that of Bt (Höfte and Whiteley 1989).

6.1.3 Bt Rice

Rice is the focus of many crop improvement programmes because it is consumed as a staple food across Asia and Africa. Moreover, the growing population of the world demands more food production. Therefore, various scientific advancements and strategies have been adopted to meet this demand. The International Program on Rice Biotechnology (IPRB) has been providing funds since 1984 for developing and creating such scientific advancements and strategies. This programme mainly supports rich research programme activities in developing countries (Datta 2004; Normile 1999). A number of genetically engineered rice varieties have been developed containing transgenes encoding Bt insecticidal proteins. These proteins kill one or more lepidopteran insect pest of rice including leaf folders, yellow stem borer and striped stem borer (Pathak and Khan 1994). Among rice insect pests, the yellow stem borer (*Scirpophaga incertulas*) is one of the most important. During wet and rainy season stem borer infestation in rice increased up to 30% (IRRI Annual Report 1999), as stem borer breeding approach is not up to the mark to control the damage. Thus scientists have engineered various local lines of rice, containing single Cry gene (1Aa, 1Ab, 1Ac, 2A and 1B) or combinations of these genes (Table 4) (Ho et al. 2006; Breitler et al. 2004; High et al. 2004). In field trials there was 80% decrease in pesticide application for Bt rice as compared to non-Bt rice, thus less labour requirement and lower exposure of farmers to hazardous effect of pesticides (Rozelle et al. 2005; Huang et al. 2015). Experimental documented reports have shown that Bt rice have no effect on aquatic ecosystem and are harmful on non-target organisms (Li et al. 2014; Niu et al. 2017). The first field trial of the Bt rice was performed by Chinese scientist in 1998, and China's Ministry of Agriculture issued biosafety certificates for two Bt rice (Huahui No. 1 and Bt Shanyou 63). Moreover, scientist have developed or been developing transgenic varieties containing insect-resistant genes. But unfortunately, none of the Bt rice has been approved and adopted at commercial level till date worldwide. The reason of delay in commercialization of Bt rice is national international biosafety regulations. Regulatory system is comprised of field trials, environmental release trials and preproduction trials. In China researchers are in continuous struggle for the last 20 years to test and investigate the effect of these transgenes on environment and non-target organisms, for the approval of commercial use (Li et al. 2014).

Table 4 Commercialized insect pest-resistant transgenic rice varieties

Developer/ trade name	Trade name	Method of trait introduction	GM trait	Commercial trait	Gene source	Gene introduced	Product	Function
Huazhong Agricultural University (China)	BT Shanyou 63	Not available	Lepidopteran insect resistance	Insect resistance	*Cry1Ab*	*Bacillus thuringiensis* subsp. *kurstaki*	Cry1Ab delta-endotoxin	Kills lepidopteran pest by selective lysis of insect midgut epithelial cell lining
					Cry1Ac	*Bacillus thuringiensis* subsp. *kurstaki* strain HD73	Cry1Ac delta-endotoxin	
Huazhong Agricultural University (China)	Huahui-1	Not available	Lepidopteran insect resistance	Insect resistance	*Cry1Ab*	*Bacillus thuringiensis* subsp. *kurstaki*	Cry1Ab delta-endotoxin	Kills lepidopteran pest by selective lysis of insect midgut epithelial cell lining
					Cry1Ac	*Bacillus thuringiensis* subsp. *kurstaki* strain HD73	Cry1Ac delta-endotoxin	
Agricultural Biotech Research Institute, Iran	Not available	Microparticle bombardment of plant cells or tissue	Lepidopteran insect resistance + antibiotic resistance	Insect resistance	*aph4* (*hpt*)[a]	*Escherichia coli*	Hygromycin B phosphotransferase (hph) enzyme	Allows selection for resistance to the antibiotic hygromycin B
					cry1Ab (truncated)	Synthetic form of *Cry1Ab* from *Bacillus thuringiensis* subsp. *kumamotoensis*	Cry1Ab delta-endotoxin	Kills lepidopteran pest by selective lysis of insect midgut epithelial cell lining

Source: ISAAA database
[a]Selection marker/reporter

6.2 How to Produce Genetically Modified Crops

The basic methodologies to be used for the production of genetically modified crops include the following steps (Jhansi Rani and Usha 2013; Fiester 2006):

1. Identification of the gene of interest with desired plant trait is the very first step of GM plant production. The gene of interest is selected using forward genetics approach using genetic basis for phenotypic appearance. This screening approach has been effectively used to understand and reveal complex developmental processes and gene regulation. Thus, a single gene with desired trait is not enough because gene regulation of this gene and its interaction with other genes are also important aspects.
   This process has been speeded up by high-throughput biology experiments that produce huge data of genes comprising genomics, proteomics and metabolomics. The generated data can be analysed using computational methods in the form of advanced and high-throughput software and bioinformatics (including online and offline). The available data for the gene in questions should fit into the larger picture. For example the National Center for Biotechnology Information (NCBI) can help to answer such types of questions. NCBI genome workbench can be used to view the data in multiple ways. Moreover, this data can be analysed together with other public databases. This workbench could be helpful in DNA sequence alignment, tubular view and phylogenetic analysis. Based on the available data of genes, we can design a synthetic gene for target plant and crop, making the gene fit for desirable expression.
2. The desired gene can be isolated with the help of polymerase chain reaction (PCR) using specifically designed primers or digestion of genomic DNA. The PCR product and fragmented DNA are then ligated into desired vector (cloning vector or expression vector). However, once the gene has been ligated and cloned, a number of modifications are made in it for efficient transformation into target plant.
3. Integrated genetic material in target plant genome. This change in plant is heritable and can be transferrable as heredity material. Although there are number of ways available to transforming plant cell and tissues, the most common methods include ballistic gun or gene gun and *Agrobacterium*-mediated plant transformation. Gene gun method is used for gene transfer to monocot plant, e.g. rice and corn. While *Agrobacterium* transformation technique involves the use of bacterium *Agrobacterium tumefaciens*, having the capability to infect plant with a fragment of DNA, this method is considered preferable to the gene gun method. This approach is mainly used for the production of insect-resistant or herbicide-resistant plants and crops.
4. After transformation the next step is screening for successfully transformed cells and tissues. The screening is made using defined medium containing antibiotics. The antibiotic will allow the growth on only those plant cells and tissues which contain the transformed gene. In the next step, the positive cells or tissues are regenerated to the whole plant using tissue culturing techniques in controlled environment containing the required growth nutrients and hormones.

6.3 Technologies for Insect-Resistant Crop Production

For hundreds of years, farmers had adopted crop varieties that offer resistance to insect pests. Conventional plant breeding technique is significantly used for the creation of insecticide-resistant plant in addition to mutagenesis and recombinant DNA technology techniques. Moreover, the introduction of new techniques of molecular biology and genetic engineering has revolutionized the agriculture by providing insect-resistant crop varieties. The genetic changes in crops and plants are not only responsible for reduced pesticide applications but also in helping in improving economic state of farmers and protecting their health. Conventional methods of pest control, i.e. extensive use of pesticides, result in insecticide resistance development in insects. By adopting these new technologies for improvement of existing crops varieties will enable scientists and breeders to develop insect-resistant varieties in a short span of time, requiring less amount of agrochemicals. There are a number of techniques used to produce insect-resistant crop varieties.

6.3.1 Mutagenesis

Mutagenesis is one of the earliest methods used for improving crop desired traits artificially. This technique involves the treatment of crops with physical mutagen (ionizing radiations, e.g. ultraviolet) and chemical mutagen or combination of both mutagens, for mutant trait development (Krishnan et al. 2009). The interaction of the mutagen with genetic material of plant results in mutation (e.g. insertion, deletion and inversion). Moreover, a desired mutation can be created with the help of using site-directed mutagenesis using specific primers using polymerase chain reaction. According to the Mutant Variety Database of IAEA, 23 different approved plant varieties resistant of insect including eight rice varieties have been created with the help of mutagenesis technique.

6.3.2 Introduction of Foreign Gene

The main strategies to develop insecticide resistant plants are based on the isolation of desired trait gene from an organism and its introduction to target crop or plant as a foreign gene. One of the common examples is the insertion of *Bacillus thuringiensis* (Bt) toxin genes to crop plants.

(a) Gene Pyramiding

This approach involves stacking of multiple genes into a single species genetic material to pool desired traits using recombinant DNA technology and conventional breeding practices. This approach is also recognized as second generation of genetically engineered crops. Gene pyramiding is done through crosses between transgenic crops with different desired genes, e.g. Agrisure™ and Viptera™ maize. The other methods involved transforming the plant multigene cassette transformation (contains two or more genes), e.g. Herculex™ maize, or introduction of one or more genes into GM plant Bollgard™ II cotton. Marker-assisted selection (MAS) is a non-transgenic technique used to find out the molecular marker linked to desired gene. These identified genes can be pyramided through crosses of different varieties (Kou and

Wang 2010). A number of virus-resistant transgenic plants have been developed, e.g. Patwin wheat.

(b) Genetically Engineered/Modified Bt Toxins

As a nature of fact that in the past control strategies for insect pest have led to the development of resistant insect. According to the Arthropod Pesticide Resistance Database, since the introduction of Bt crops (1996–2011), 5 out of 13 major pest species have developed resistance. Other issues related to the transgenic insect-resistant crops include; patent expiration, which will lead to seed saving by farmers for future use and improper replant practices. Another threat to these transgenic crops is transgene escape. There is a need to avoid practices of saving engineered seeds, which could lead to revision of the international moratorium on genetic use restriction technologies (GURTs). As discussed above Bt toxin requires proteolytic cleavage to became activated with the help of insect gut protease enzyme. This step is one of the limiting factors for the effective application of Bt toxin. As a matter of fact, Cry proteins share identity in their domain structure and conserved regions. Scientists have explored that modification or truncation of protein will not affect its toxic activity (Deist et al. 2014). This approach is working as an alternative mechanism for Bt toxicity. For example, truncation of Cry1Ab and Cry1Ac proteins resulted in enhanced toxicity against Bt-resistant lepidopteran insects 19, 20. Similarly, modified Cry3A toxin offers great resistance to western corn rootworm *Diabrotica virgifera virgifera* (Walters et al. 2008). Furthermore, the chimeric toxin eCry3.1Ab was developed by fusing domains I and II of Cry3Aa with domain III of Cry1Ab. This new toxin was highly bioactive against the larvae-resistant western corn rootworm (Walters et al. 2010). However, one of the drawbacks of truncated Bt toxins is that it can affect natural enemies of the insect.

As documented that insect pests have developed resistance to Bt toxins, pyramiding strategies and combining natural and engineered Bt toxins can be utilized to expand the range of options for pest control (Tabashnik et al. 2013).

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Increasing Rice Grain Yield Under Biotic Stresses: Mutagenesis, Transgenics and Genomics Approaches

Aamir Raina and Samiullah Khan

Abstract

Rice (*Oryza sativa* L.) is the most important source of staple food to a major portion of human population. The production of rice is reduced by several kinds of biotic stresses. The main biotic stresses that severely hamper the rice production include viruses, bacteria fungi, nematodes and insects. Different conventional and modern biotechnological approaches have been implemented to combat the devastating effect of different biotic stresses on the rice production. Conventional approaches such as hybridisation have led to the development of stress-tolerant varieties. The modern biotechnological approaches such as genomics and transgenics have led to the identification of genes that confer tolerance to stresses followed by its insertion into the rice plants with the aim of decreasing the yield loss incurred by the different stresses. Mutagenesis, genomics and transgenic approaches have been very effective in developing varieties with improved tolerance to various stress factors. Here we review the creation of rice varieties with improved yield under different biotic stress, using mutagenesis, transgenics and genomics approaches.

Keywords

Biotic stresses · Breeding techniques · Yield · Stress tolerance · Mutagenesis · Transgenics · Genomics

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1 Introduction

Oryza sativa commonly known as rice belongs to family Poaceae with more than 80,000 accessions maintained at International Rice Research Institute (IRRI), Philippines. It is an ancient staple food with the origin of centre in southern and south-western tropical Asia and origin of domestication in India and China (Vavilov 1926; Ding 1957). *Oryza sativa* is considered as the main cultivated species of rice across the globe. It is one of the main cereal grains and source of food for more than 3.5 billion people, grown on 145 million ha in more than 110 countries (IRRI, Africa Rice and CIAT 2010; Heinrichs 1994). With the rapid increase in human population which is expected to increase up to 9 billion, by the end of 2050, rice production must increase by substantial amount. Increasing population and economic development have been posing a growing pressure for increase in rice production (Zhang 2007). This increase in rice production is a challenging task due to several factors such as decrease in rice lands, depleting water resources, erratic rainfalls and climate change. Further the overall yield of rice is sternly reduced by several biotic stress factors including virus, bacteria, fungi, insect pest, nematodes and diseases (Shamim and Singh 2017). To meet the challenges new rice varieties with improved yield and better tolerance to biotic stresses should be developed. This can be achieved by making the use of modern biotechnological approaches. Rice production needs to increase via biotechnological techniques with the objective of improving yield, resistance to biotic stresses and grain quality (Shamim and Singh 2017). Here we review the current progress in the field of mutagenesis, transgenics and genomics for the development of rice varieties that are resistant to wide range of biotic stresses.

1.1 Biotic Stresses

Rice production is negatively impacted by a wide range of biotic stresses that cause dreadful diseases and significantly decrease the overall productivity by 30% (Yadav and Srivastava 2017). Biotic stresses that devastate the rice production include virus, bacteria, fungi, nematode and insect pests (Ling 1980). Conventional breeding approaches have been implemented to combat the effects of biotic stresses but all such approaches have some limitations. Few limitations include cumbersome, laborious and huge time taken usually 10 years for the release of varieties with improved tolerance and yielding potential. Different causative agents results in the occurrence of dreadful diseases that incur huge loss in both production and economic values. On an average 10–15% of annual yield is lost due to different rice diseases across the world. In India different causative agents cause a substantial decrease in rice production that range from 6 to 60% depending upon the growth stage, variety and timing of occurrence of stress (Ou 1985; Singh et al. 1977). Hence, proper disease management could be useful to enhance production and recovery of yield losses. The rice diseases that have incurred a huge economic losses are rice blast (causative agent: *Magnaporthe grisea*), seedling blight (causative agent:

Pseudomonas plantarii), sheath blight (causative agent: *Rhizoctonia solani*), bacterial blight (causative agent: *Xanthomonas oryzae*), bacterial brown stripe (*Pseudomonas avenae* and *P. syringae* pv. *panici*), tungro virus disease and false smut (FS) (causative agent: *Ustilaginoidea virens*). Modern breeding approaches such as mutagenesis, transgenics and genomics have proven promising techniques in developing varieties with improved biotic stress tolerance. Among the techniques RNA interference (RNAi)-induced gene silencing has proven as an effective and efficient technique to engineer resistant plants to various kinds of biotic stresses and to mediate management of rice diseases. Rice is continuously affected by various organisms from insects to bacteria. A study estimated an annual loss of yield ranging from 120 to 200 mt due to wide range of causative agents in rice lands of tropical Asia (Willocquet et al. 2004). Biotic stresses that affect the rice production are discussed in this chapter.

1.2 Viral Diseases

Viral diseases represent a severe threat to rice production in Southeast Asian countries. The most common symptoms include abnormal growth and colour changes on leaves from green to yellow to white/orange. The teratological symptoms are stunted growth, reduced tillers, twisting, leaf rolling, gall formation on leaves and necrotic spots on culms. The rice yellow mottle virus (RYMV) is one of the most detrimental virus infecting rice. Rice tungro disease (RTD) is another damaging disease of rice, widespread in South and Southeast Asia. RTD incurs an annual loss of about 109 US dollars in the affected countries (Herdt 1991) and about 2% reduction in overall production in India (Muralidharan et al. 2003). A DNA virus, viz. *Rice tungro bacilliform virus* (RTBV), and an RNA virus, viz. *Rice tungro spherical virus* (RTSV), are causative agents of rice tungro disease. The initial reports of appearance of RTD in India came into notice in the late 1960s (Raychaudhury et al. 1967a, b), and thereafter extensive studies were carried out for its management (Rivera and Ou 1965). At present new information on theoretical and practical aspects and diagnostic techniques consistently regarding the causative agents, pathogenesis, vector transmission and resistance genes of RTD became available and sophisticated over time (Azzam and Chancellor 2002). In general plant viruses are transmitted mechanically and/or by means of vectors such as insects, mites, nematodes, fungi, dodders, pollen, seed, grafting, budding, vegetative propagation or soil (Sasaya 2015).

1.3 Bacterial Diseases

Bacterial diseases are the most devastating diseases of rice, found in tropical and temperate regions of the world, which include bacterial blight, leaf streak, foot rot, grain rot, sheath brown rot and pecky rice. Rice bacterial leaf blight (BLB), caused by *X. oryzae* pv. *oryzae* (*Xoo*), a Gram-negative bacterium, is one among the

severely damaging diseases in rice (Ishiyama 1922). The outbreak of BLB as a seed-borne disease was first reported in 1884 at Japan (Saha et al. 2015). All the phases of growth are negatively impacted by BLB infection under favourable environmental conditions. However, rainy season and fast winds exaggerate the epidemic of BLB and result in further damage. The decrease in production caused by BLB range from 20 to 30% and can reach up to 80% in some of the cultivated area under severe infection (Chattopadhyay et al. 2017). Symptoms include yellow or white stripes on leaf blades, grayish leaves, wilting and stunted growth and plant death (Agrios 2005). The production of rice varieties with enhanced tolerance to bacterial disease is the efficient and sustainable approach for the management of disease, even though detection and subsequent selection of resistant source through screening under high pressure of BLB have been effectively exploited for the creation and release of resistant varieties. However, the co-evolution of new virulent mutant strains of *X. oryzae* pv. *oryzae* has always been a challenge for BLB resistance rice breeders. The recent advancements in the modern breeding approaches such as genomics, MAS and transgenics new genes that govern the resistance to bacterial blight have been identified, characterised, cloned and transferred to improve resistance into rice breeding. Compared to single gene introgression, pyramiding of multiple genes via MAS strategy has proven effective for disease management. Nonetheless, some of the advanced transgenic approaches such as overexpression, silencing and knockout of genes, genome editing techniques like TALEN (transcription activator like effector nucleus) and CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein) are also being employed in the recent past to develop complete resistance against this highly damaging bacterial disease (Mishra et al. 2018).

1.4 Fungal Diseases

Several species of fungi infect most important agricultural crops including rice and cause a significant reduction in overall production. Fungal diseases are considered as primary biotic stress that contributes to huge loss in rice yield (Srivastava et al. 2017). Agrios (2005) reported that about 70% of all major crop diseases are caused by fungi. The severely damaging fungal diseases of rice reported till now are "blast", "heliminthosporiose", "stem rot" and "foot rot", of these "blast" disease is more devastating and prevalent. Among the diseases of rice, false smut (FS) caused by *U. virens* decreases yield to a great extent. Recently *U. virens* has been placed in Clavicipitaceae and renamed as *Villosiclava virens* (Teleomorph) (Kepler et al. 2012; Tanaka et al. 2008), based on its ability to reproduce by both sexual and asexual means (Fu et al. 2012; Singh and Dubey 1984). The increased progress of FS in rice-growing area has been attributed to the use of nitrogen fertilisers and cultivation of hybrids on larger-scale cultivars (Deng 1989). Another ascomycete fungus *Magnaporthe oryzae* that causes a severe disease called as rice blast is the widespread in all rice-growing nations and led to 60–100% reduction in yield (Kihoro et al. 2013; Zhang et al. 2014).

1.5 Nematode Diseases

Plant–parasitic nematodes devastate the crops worldwide and pose a serious threat to the overall crop production (Raina et al. 2019a; Raina and Danish 2018). Among the biotic stresses, plant–parasitic nematodes represent another severe threat to the rice production (Soriano et al. 1999). As per Bridge et al. (2005), plant parasitic nematodes cause 10–25% yield losses annually worldwide, and economic loss corresponds to a monetary value of US$16 billion. Plant–parasitic nematodes attacks roots of herbs, shrubs and trees and upon infection reach to the aerial shoots and can feed on internal tissues (Soriano et al. 2004). Till now 150 species of plant–parasitic nematodes are known that can cause severe reduction in overall yield of rice due to very effective dispersal means viz. wind, water, animals and infected plant propagules. Among the different plant–parasitic nematodes, *Meloidogyne* spp. belong to a group of root-knot nematodes (RKNs), associated with root of crops, and induce gall formation in rice roots (De Waele and Elsen 2007), represented by more than 90 species (Moens et al. 2009). This RKN species is an obligate sedentary endoparasite that settles in roots and completes their entire life cycle inside the root cells and causes extensive damage to growth and development of rice (Williamson and Gleason 2003). The reduction in production increases when the soil is alternating dry and flooded under rain-fed conditions; therefore, water management practice influences the progress of disease (Prot and Matias 1995; Tandingan et al. 1996). In India, the first reports of RKN *M. graminicola* infecting rice were reported from Orissa (Patnaik 1969) and were equally prevalent on upland or lowland rice regions. In India, *M. graminicola* is widespread, and one of the dreadful nematode as is evident by its outbreak that devastated about 1500 ha cultivated land in Karnataka (Prasad and Varaprasad 2001).

1.6 Insect-borne Diseases

Among various obstacles in achieving the desired goals of rice production, insects incur about 30–40% of production loss. The agro-climatic conditions favourable for rice production are also conducive for rapid multiplication of insect pests (Heinrichs 1994). Infestation by insects, particularly stem borer, planthopper, leafhopper, gandhi bug, gall midge, rice leaffolder, rice hispa, cut worms and army worms is a serious challenge to achieve the desired goals of rice production (Pathak and Dyck 1973; Lou et al. 2013). However, the major insects that cause substantial reduction in rice yield include planthopper and leafhopper which cause direct damage and facilitate rapid transmission of viral diseases (Heinrichs 1994). About 100 insect pest species infest and damage the rice plant, among them 20 insect pests represent a serious threat to the production (Heinrichs 1994). The main stem borer species attacking every stage of growth include *Scirpophaga incertulas* (yellow stem borer) and *Sesamia inferens* (pink stem borer) and *Chilo polychrysus* in rice lands of Asia (Banerjee 1971; Pathak and Khan 1994). The degree of borer-caused reduction in rice yield has been estimated to range from 2 to 20% in non-outbreak

per year and 30 to 70% in outbreak per year in India (Chelliah et al. 1989; Satpathi et al. 2012) and in Bangladesh (Catling et al. 1987), respectively. The estimated worldwide losses in rice production due to insect damage have been reported as 34.4% (Cramer 1967). Brown plant hopper (BPH) is also considered as the most serious damaging pests to the rice crop globally as they cause direct damage and also act as vectors for several dreadful viruses especially in rice lands with heavily fertilised soils. Chemical fertilisers and insecticide have been implemented to control the propagation of insect pests, but the limitation is the deterioration of grain quality. Hence, it is imperative to create rice cultivars with improved resistance to the insect pests. At IRRI researchers reported that rice fields protected from insects yielded almost double than unprotected rice fields and showed the impact of the insect pests on the overall rice production (Heinrichs 1994). Rice breeding programs have gained much success in the selection for insect-resistant rice varieties which showed less effect of borers' on the overall production (Khan et al. 2005). However, complete resistance against the YSB in cultivated rice varieties is still lacking and had also delayed the creation of resistant rice varieties (Bentur 2006). With the advancement in modern technology, breeding of insect pest stress tolerance have been improved by the identification, isolation and characterisation of genes that confer resistance to insect pest stresses. These genes can be introduced in rice varieties with higher yield but sensitive to insect borne diseases. The advancement in rice transgenic technologies has paved a way for the development of genetically modified (GM) rice that showed increased tolerance to insect pests (Bhattacharya et al. 2006).

2 Modern Breeding Approaches to Combat Biotic Stresses

The conventional breeding approaches have proven inefficient in improving the tolerance to biotic stress factors. To overcome the limitations of conventional breeding strategies, modern breeding approaches, viz. mutagenesis, transgenics and genomics, are employed for the creation of varieties with enhanced resistance to biotic stresses (Shamim and Singh 2017). At present, a collaborative research is going on to identify multiple stress factors involved in biotic stress tolerance and is discussed in detail in the following subsections.

2.1 Mutagenesis

In mutagenesis different chemical mutagens such as ethyl methane sulphonate, methyl methane sulphonate, sodium azide, hydrazine hydrates and physical mutagens such as gamma rays, X-rays, UV rays, heavy ion beams and laser beams are used by plant breeders to create rice genotypes with increased yield and better tolerance to biotic stresses (Raina et al. 2016; Khursheed et al. 2019). Among different breeding approaches, mutagenesis has proven to be a very effective tool for enhancing the genetic variation and improving resistance to biotic stresses. Additionally, mutagenesis equips the plant breeders to make the efficient selection

of the desired genotype (Raina et al. 2018a Goyal et al. 2020). De Vries (1901) has first conceptualised the use of mutations for developing novel varieties in crops. Later on Stadler (1928) while working on barley has documented the practical significance of mutation breeding. Muller (1927) and Stadler (1928) and Ganger and Blakeslee (1927) were pioneers in authentication of use of electromagnetic waves in increasing the frequency of mutations in *Drosophila*, *Zea mays* and *Datura*, respectively. The first mutant Chlorina, mutant of *Nicotiana tabacum* was developed through the X-ray irradiation of floral buds in the 1930 (Coolhaas 1952). The collaborative research of FAO/IAEA lead to extensive and systematic research on use of mutations for the improvement of traits in wide range of crops. Several workers have employed mutation breeding for the improvement of different traits in different crops (Khursheed et al. 2015; Amin et al. 2016; Kalapchieva and Tomlekova 2016; Raina et al. 2019) that considerably reported the efficacy of induced mutations in crop improvement. Several researchers have employed different mutagens in different doses for creating varieties with desired traits in crops like lentil (Laskar et al. 2018a, b), cowpea (Raina et al. 2018b, 2020), mungbean (Wani et al. 2017), urdbean (Goyal et al. 2019a, b), fenugreek (Hasan et al. 2018), chickpea (Laskar et al. 2015; Raina et al. 2017, 2019b), black cumin (Amin et al. 2016, 2019; Tantray et al. 2017) and faba bean (Khursheed et al. 2018a, b, c). The plant traits improved by mutation breeding include yield, earliness, adaptability and tolerance to viral, bacterial, fungal and insect pests attack (Aetsveit et al. 1997; Khursheed et al. 2015, 2016; Laskar et al. 2019).

Mutation breeding in rice has been successful in developing and officially releasing 130 rice mutant varieties with improved traits like high yield, better grain quality and better resistance to biotic stresses. Earlier, the identification of mutated genes in subsequent generations was not possible due to lack of sophisticated biotechnological tools. In the recent years, a huge advancement in the modern breeding tools has led to the easy identification, isolation and transfer of newly mutated genes into stress susceptible variety without modifying the whole genome (Shu 2009). The possibilities of mutagenesis include development of new alleles and their incorporation into the new varieties that can be later on released as a commercial variety. Several attempts have been made to enhance tolerance to biotic stress in many crops including rice through mutagenesis. Mutant lines such as Camago-8, Heiseimochi, ITA 235, Shengba-simiao, Zhe 101, Zhengguang 1 and Zhongzao 21 have been developed that showed resistance to various viral diseases (Table 1) (mvd.iaea.org accessed July, 2019). Mutagenesis has also led to the development of 25 rice mutants that showed better resistance to bacterial diseases (Table 1) (mvd.iaea.org accessed July, 2019). Similarly mutation breeding has been successful in developing 100 mutant varieties of rice with enhanced tolerance to fungal diseases (Table 1) (mvd.iaea.org accessed July, 2019). The rice mutant variety named as RD6 has been developed by irradiating the non-glutinous variety Khao Dawk Mali 105 (KDML 105). The mutant variety showed promising results in terms of resistance to blast (*P. oryzae*) (Khambanonda 1978). The EMS dose of 0.1 and 0.2% concentrations was employed to develop blast resistance in the rice variety HYV Ratna (IR8/TKm 6). Few mutant lines in the M2–M5 generations showed better

Table 1 Role of mutagenesis in improving tolerance of rice to biotic stresses

Name	Country	Year	Mutagen (dose)	Improved trait (s)
Zhengguang 1	China	1978	Gamma rays (300 Gy)	Yellow stunt virus
Camago-8	Costa Rica	1996	Gamma rays (250 Gy)	Resistance to blast and resistance to viruses
Heiseimochi	Japan	1988	Gamma rays (250 Gy)	Resistance to rice stripe virus
ITA 235	Nigeria	1988	Chemical mutagen	Semi-dwarfness and resistance to viruses (RYMV)
Zhongzao 21	China	2003	NA	Large spike and more grains, blast resistance
Shengba-simiao	China	2005	NA	Resistance to viruses
Zhe 101	China	2005	NA	Late maturity, high yield, resistance to blast and bacterial blight
Fulianai	China	1966	Gamma rays (200 Gy)	Short culm, resistance to blast, early maturity and high yield
Yangfuxian 2	China	1991	Gamma rays (300 Gy)	Resistance to bacterial diseases, high grain yield and good quality
Fuchuerai	China	1978	Gamma rays (350 Gy)	Shorter culm and improved resistance to bacterial leaf blight
Fuxian 6	China	1989	Hybridisation with mutant Fu 774	Early maturity (107–110 days), earlier and higher yielding, good resistance to BLB
Zhe 852	China	1989	Gamma rays (200 Gy)	Resistance to bacterial diseases, stress resistance, good grain quality characteristics and high grain yield
Zhefu 9	China	1990	It was developed by direct treatment with mutagen (IR50/44-1086)	Resistance to bacterial diseases, high yield
Yangfuxian 3	China	1993	Gamma rays (300 Gy)	High yield and resistance to bacterial diseases
Xiangzaoxian 21	China	1996	Combined treatment with gamma rays (288 Gy) and He-Ne laser	High yield, early maturity and resistance to bacterial diseases
Yuanjing 7	China	1999	Gamma rays (300 Gy)	High grain yield and resistance to bacterial diseases

(continued)

Table 1 (continued)

Name	Country	Year	Mutagen (dose)	Improved trait (s)
Xiangzaoxian 25	China	1997	Developed by hybridisation with one mutant Fu 26	High grain yield and resistance to bacterial diseases
Atomita 3	Indonesia	1990	Gamma rays (200 Gy)	Tolerance to brown plant hopper, BLB and bacterial leaf stripe, high yield
DB 250	Vietnam	1987	Gamma rays (250 Gy) and with 0.020% MNH during 6 hours	Resistance to lodging, resistance to bacterial blight and *Pyricularia oryzae* and yield (4.5 t/ha)
DT-10	Viet Nam	1989	Gamma rays (200 Gy) and with 0.025% MNH	Resistance to bacterial leaf blight and insects
Yangfuxian 9850	China	2004	Gamma rays (300 Gy)	High yield, good resistance to bacterial leaf blight, blast, sheath blight light
Yangfujing 4298	China	2004	NA	Improved agronomic traits and resistance to bacterial diseases
Yangfujing 4901	China	2004	Gamma rays	Strong resistance to blast, bacterial leaf blight, lodging resistance
Zhenuo #3	China	2003	Gamma rays	High yield and tolerance to bacterial diseases
Chiyou S162	China	2005	Gamma rays (300 Gy)	Improved yield, resistance to blast and bacterial blight
Nanhua 11	China	1987	Carbon dioxide laser irradiation of callus	High yield, resistance to bacterial diseases
Yangfuxian 5	China	2000	Gamma rays	High quality, high yield, multiple resistance
Kahayan	Indonesia	2002	Gamma rays (200 Gy)	High yield, resistance to leaf blight and amylose content (19–20%)
Winongo	Indonesia	2002	Gamma rays (200 Gy)	High yield, resistance to leaf blight and amylose content (19–20%)
Diah Suci	Indonesia	2003	Gamma rays (200 Gy)	High yield, resistance to leaf blight and amylose content (19–20%)
Mira 1	Indonesia	2006	Gamma rays (200 Gy)	High yield and resistance to bacterial diseases

(continued)

Table 1 (continued)

Name	Country	Year	Mutagen (dose)	Improved trait (s)
Aifu 9	China	1966	Gamma rays (300 Gy)	Short culm, resistance to blast and higher yield
Fuwan 23	China	1978	Gamma rays (300 Gy)	Resistance to yellow stunt and *Xanthomonas*, bigger spike, large grain size
Fuxuan 3	China	1970	Gamma rays (300 Gy)	Good tillering and resistance to blast
Fuxuan 124	China	1972	Gamma rays (300 Gy)	Resistance to blast
Jinfu 1	China	1969	Gamma rays (300 Gy)	Early maturity and resistance to blast
Kefuhong 2	China	1981	Developed by hybridisation with mutant IR8	Early maturity and resistance to blast
Wanfu 33	China	1978	Gamma rays (300 Gy)	Early maturity, resistance to blast
Wangeng 257	China	1975	Gamma rays (300 Gy)	Tolerance to fertilisers, resistance to blast and higher yield
Xiangfudao	China	1976	Gamma rays (300 Gy)	Resistance to blast and *Xanthomonas*
Xiongyue 613	China	1965	Gamma rays (200 Gy)	Moderate resistance to blast, higher yield and good quality
Yifunuo 1	China	1973	Gamma rays (100 Gy)	Resistance to blast, bigger spike and higher grain number
Fulianzao 3	China	1968	Gamma rays (300 Gy)	Early maturity, resistance to disease and short culm
Fushe 410	China	1974	Gamma rays (300 Gy)	Intermediate resistance to blast
Fu 769	China	1976	Gamma rays (300 Gy)	Resistance to diseases and high yield
Fu 756	China	1975	Gamma rays (300 Gy)	Resistance to diseases, good taste
M 112	China	1981	Gamma rays (300 Gy)	Resistance to *Sogatella furcifera* and high yield
Wanhongfu	China	1980	Gamma rays (350 Gy)	Resistance to low temperature and resistance to diseases
Zhuqin 40	China	1978	Gamma rays (300 Gy)	Resistance to blast
240	China	1980	Gamma rays (300 Gy)	Early maturity and resistance to diseases
Fushenongken 58	China	1973	Gamma rays (300 Gy)	Resistance to fungal diseases and high grain yield

(continued)

Table 1 (continued)

Name	Country	Year	Mutagen (dose)	Improved trait (s)
Ejingnuo 6	China	1986	Gamma rays (350 Gy)	Resistance to blast and blight, good grain quality and higher grain yield
Erjiufeng	China	1982	Gamma rays (350 Gy)	Higher yield, early maturity and resistance to fungal diseases
Taifu 4	China	1979	Gamma rays (200 Gy) and colchicines	Resistance to diseases and low application of fertilisers
652	China	1979	Gamma rays (300 Gy)	Resistance to fungal diseases
Xiushui 48	China	1981	Developed by hybridisation with mutant Funong 709	Resistance to blast, tolerance to low temperature and high yield
Xianghu 24	China	1983	It was developed by hybridisation with mutant Funong 709 [(Funong 709 × Jingyin 154) × Funong 709]	Resistance to blast and blight and glutinous grain type
Ailiutiaohong	China	1989	Gamma rays	Dwarfness (88 cm), high yield, resistance to *Pyricularia oryzae*, resistance to insects
Qingwei 1	China	1985	Gamma rays	High yield, resistance to diseases and late maturity
Fu 8-1	China	1988	Gamma rays (350 Gy)	Resistance to fungal diseases and high grain yield
Tangernian	China	1985	Gamma rays	High yield, resistance to diseases and late maturity
Wanhua	China	1983	Gamma rays (350 Gy)	Semi-dwarfness, resistance to diseases, superior grain quality and high yield
Fuwan 81-548	China	1989	Gamma rays (300 Gy)	Good quality and resistance to fungal diseases
Meisanwu 2	China	1990	Gamma rays (150 Gy)	Resistance to fungal diseases and insects
Xiuxui 117	China	1984	It was developed by direct use of mutagen treatment on Funong 709/Zaison/Funong709/ Chengbaoxifeng	Resistance to fungal diseases and altered maturity

(continued)

Table 1 (continued)

Name	Country	Year	Mutagen (dose)	Improved trait (s)
Xianghu 93	China	1984	(Funong 709 × Jingyin 154) × Funong 709	Resistance to fungal diseases and altered maturity
Zijiangnuo	China	1984	(Fuhong 3 × Xinbasi × Nenjing 15 gamma)	Resistance to fungal diseases and high grain yield
Xiushui 04	China	1985	(Ze 21/Funong 709/Dan 209)	Resistance to fungal diseases, blast and bacterial blight, high yield
Ganwannuo	China	1993	Developed by hybridisation with one mutant MY82166	High grain yield and resistance to fungal diseases
Wandao 20	China	1994	Ion beams	Altered maturity and resistance to fungal diseases
Wandao 45	China	1994	Ion beams	Altered maturity and resistance to fungal diseases
Shenxiangjing	China	1994	NA	Improved plant structure and resistance to fungal diseases
Zhefu 762	China	1993	NA	High grain yield (5–10%), high resistance to blast, bacterial blight resistance
Ganwanxian 23	China	1994	It was developed by hybridisation with one mutant (TR 841 × M79215)	High quality and resistance to fungal diseases
Fuxuan 8	China	1998	(Fu 8329 × Fu 8105 × IR13471–74-1)	Resistance to fungal diseases
Camago-8	Costa Rica	1996	Gamma rays (250 Gy)	Resistance to blast and resistance to viruses
Camago-8	Costa Rica	1996	Gamma rays (250 Gy)	Resistance to blast and resistance to viruses
UNP 9027	Costa Rica	1994	Gamma rays (200 Gy)	Resistance to *Pyricularia oryzae*
IRAT 216	Cote D'Ivoire	1985	Gamma rays	Good adaptability to wetland rice culture, resistance to *Pyricularia*
Calendal	France	1979	Gamma rays	Longer grains, improved trashability, resistance to *Sclerotinium oryzae*

(continued)

Table 1 (continued)

Name	Country	Year	Mutagen (dose)	Improved trait (s)
Marathon	France	1985	Gamma rays	Resistance to *Pyricularia*
Nucleoryza	Hungary	1972	Fast neutrons (25 krad)	Early maturity, maintained blast resistance and improved yield
Mutashali	Hungary	1980	Fast neutrons (20 Gy)	Resistance to *Pyricularia oryzae* and high yield
Pusa-NR-381	India	1989	Gamma rays	Resistance to blast
CRM 49	India	1999	0.001 M sodium azide (NaN3)	Resistance to blast disease
CRM 51	India	1999	0.001 M sodium azide (NaN3)	Resistance to blast disease
CRM 53	India	1999	0.66% EMS	Resistance to blast disease
Atomita 1	Indonesia	1982	Gamma rays (200 Gy)	Early maturity, resistance to BPH, GLH and blast
Danau atas	Indonesia	1988	Gamma rays (400 Gy)	Resistance to blast, high yield
Fulgente	Italy	1973	X-rays (250 Gy)	Blast resistance and high productivity
Sachiminori	Japan	1978	Gamma rays	Stiff culm and resistance to blast
ITA 123	Nigeria	1980	Gamma rays (20–2000 Gy)	Semi-dwarfness and resistance to rice blast
RD 6	Thailand	1977	Gamma rays (200 Gy)	Glutinous endosperm and improved resistance to blast
Pooya	Iran, Islamic Republic of	2004	Gamma rays (150 Gy)	Resistance to lodging, resistance to blast and higher yield
Tabesh	Iran, Islamic Republic of	2004	Gamma rays (150 Gy)	Resistance to lodging, short culm, tolerance to blast and higher yield
Minnuo 706	China	1988	Gamma rays (250 Gy)	Good tillering, higher yield, glutinous, resistance to blast, good quality
Jinhang-simiao	China	2006	Aerospace	Resistance to fungal diseases and good quality
Huahang-simiao	China	2006	Aerospace	Resistance to fungal diseases and good quality

(continued)

Table 1 (continued)

Name	Country	Year	Mutagen (dose)	Improved trait (s)
Peiza 130	China	2008	Aerospace	High yield, resistance to fungal diseases and early maturity
Liangyouhang 2	China	2008	Aerospace	High yield, resistance to fungal diseases, blast and bacterial blight and good grain quality
Hangxiang 18	China	2008	Aerospace	Late maturity and resistance to fungal diseases
Yuanjing 41	China	2004	NA	Resistance to fungal diseases
Zhenuo 5	China	2004	NA	Resistance to fungal diseases
Yuanjing 35	China	2005	NA	Resistance to fungal diseases
Guangyinruanzhan	China	2008	Physical mutagen	High yield, high quality, resistance to blast and bacterial leaf blight
Early Samba	India	2000	It is a mutant from BPT-5204	Dwarfness, white MS grains, tolerance to SB, yield (60–65 Q/ha)
IACuba 28	Cuba	2001	Fast neutrons (20 Gy)	Large grain size, high yield, resistance to blast
Michinoku-wase	Japan	1988	Gamma rays (200 Gy)	Resistance to leaf blast
Okini-iri	Japan	1996	Gamma rays (200 Gy)	Superior eating quality and high field resistance to blast
Hayatsukushi	Japan	1997	Gamma rays (200 Gy)	Extremely early-maturity and highest field resistance to blast
Hiroshima No. 21	Japan	1998	Gamma rays (200 Gy)	Resistance to leaf and panicle blast
Koshihikari Toyama BL No. 2	Japan	1998	Gamma rays	Resistance to fungal diseases
Aichi-no-kaori SBL	Japan	1999	Gamma rays (200 Gy)	High resistance to rice stripe disease and panicle blast and BLB
Fusa-no-mai	Japan	2000	Gamma rays (200 Gy)	Suitable for sake brewing, high cold resistance, high resistance to the panicle blast
Koshihikari Niigata BL No. 4	Japan	2002	Gamma rays (200 Gy)	Resistance to blast
Koimusubi	Japan	2002	Gamma rays (200 Gy)	Excellent cultivation characteristics, blast resistance

(continued)

Table 1 (continued)

Name	Country	Year	Mutagen (dose)	Improved trait (s)
Churahikari	Japan	2003	Gamma rays (200 Gy)	High resistance to blast, medium-late maturity, shorter culm
Sai-no-kagayaki	Japan	2002	Gamma rays (200 Gy)	Field resistance to blast and stripe disease and green rice leaf hopper
Zhejing 41	China	2009	NA	Resistance to blast, bacterial leaf blight and brown plant hopper
Moretsu	Japan		Chemical mutagen MNU	High resistance to lodging and high resistance to stripe rust
M 114	China	1981	Gamma rays	Tolerance to low temperature and resistance to Fulgorid plant hopper
Meisanwu 2	China	1990	Gamma rays (150 Gy)	Resistance to fungal diseases and resistance to insects
Pusa-NR-555-5	India	1990	Gamma rays	Resistance to pests and resistance to diseases
Pusa-NR-570-17	India	1990	Gamma rays	Resistance to pests and resistance to diseases
Pusa-NR-519	India	1990	Gamma rays	Resistance to pests and resistance to diseases
Atomita 3	Indonesia	1990	Gamma rays (200 Gy)	Resistance to brown plant hopper resistance to BLB, bacterial leaf stripe, high yield
VN24-4	Viet Nam	2009	Developed by hybridisation with female variety IR64 and male mutant variety VND95-19	Bigger panicles, stiff culms, strongly seedling vigour, high tolerance to pest and diseases (BPH & GSV)

Source: mvd.iaea.org (MVD-2019)

resistance to blast disease (Kaur et al. 1971). Attempt to develop blast resistance via the use of 100 Gy gamma rays in the F1 progeny lead to the isolation of mutant R917 with improved resistance (Zhang et al. 2003). Similarly, the Mtu 17 blast-resistant mutants with elite agronomic traits were developed through chemomutagenesis with diethyl sulphate (dES) (Gangadharan and Mathur 1976). Mohamad et al. (2006) and Azlan et al. (2004) have reported several blast-resistant mutant lines, such as Mahsuri Mutant SPM 129, SPM 130 and SPM 142, which have been developed in Malaysia. Another mutant variety "Zhefu 802" with high resistance to rice blast has been developed through gamma irradiation of variety "Simei No. 2"

(Ahloowalia et al. 2004; Shu et al. 1997). Mutagenesis has also been successful in the development of rice mutant varieties which showed enhanced resistance to the insect pest attack. For instance, the varieties such as Atomita 3, M 114, Meisanwu 2, Pusa-NR-519, Pusa-NR-555-5, Pusa-NR-570-17 and VN24-4 have been developed to mitigate the effect of insect pest attack (Table 1) (mvd.iaea.org accessed July, 2019). However, mutagenesis is under progress to develop rice varieties with improved resistance to nematodes, and till date no variety of rice with tolerance to nematode attack has been developed.

2.2 Transgenics

Many morpho-physiological and biochemical traits linked with disease resistance are governed by different sets of genes. Molecular breeding have been employed for the creation of varieties with improved resistance by insertion of new resistance genes into promising lines. Conventional breeding approaches such as selection and hybridisation have resulted in the development of new varieties that can persist under pest and pathogen attack, but these approaches are cumbersome and require long duration of time. This necessitates the implementation of new and effective strategies for disease management and development of varieties with enhanced resistance to wide range of biotic stresses (Collard and Mackill 2008; Hasan et al. 2015). Modern biotechnological tools have proven very effective in enhancing the yield and reducing the crop loss due to single and/or multiple biotic stresses (Onaga and Wydra 2016). The advent of transgenics and single-gene approach where stress-responsive genes are overexpressed in stress-sensitive plants, have paved a way for the quick and efficient development of cultivars with improved tolerance to biotic stresses. Even though insecticides have been effective in controlling the viral disease, the high prices of insecticide and its environment hazard are the main demerits. Hence, transgenics wherein genes that confer tolerance to stress are introduced and overexpressed in stress-susceptible varieties have proven effective in curbing the virus infestation. Sasaya et al. (2013) developed transgenic rice with improved resistance against two tenuiviruses by introduction of double-stranded RNA. The results showed increased resistance to *rice stripe virus* (RSV) and *rice dwarf virus* (RDV) infection in transgenic rice plants induced by different RNAi-targets of RSV and RDV genes (Table 2). Another dreadful viral diseases of rice is caused by rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) with the help of a vector *Nephotettix virescens* (green leafhopper) that facilitates its quick transmission from infected to non-infected plants. At present efforts are being made by employing coat protein-mediated resistance strategy wherein rice plants have been transformed by the insertion of RTSV replicase gene. The results revealed that transformed rice were more resistant to RTSV and also showed improvement in yield (Huet et al. 1999).

Song et al. (1995) developed transgenic rice by the insertion of *Xa21* gene. Transgenic rice plants with *Xa21* revealed enhanced tolerance to bacterial blight and manifold increase in yield and yield attributed traits due to least damage caused

Table 2 Role of transgenics in improving the resistance of rice crop against biotic stresses

Gene(s)	Trait	References
cry1Ac and *CpT1*	Insect resistance	Han et al. (2006)
cry1Ab	Insect resistance	Wang et al. (2014)
Xa21	Bacterial blight resistance	Tu J et al. (2000a)
Bar	Sheath blight disease	Uchimiya et al. (1993)
Chi 11	Sheath blight disease	Lin et al. (1995)
TLP-D34	Sheath blight disease	Datta et al. (1999)
RC 7	Sheath blight disease	Datta et al. (2000, 2001)
pinA, pinB	Sheath blight disease	Krishnamurthy et al. (2001)
Chi, Xa21, Bt	Sheath blight disease	Datta et al. (2002)
ChiC	Fungal disease resistance	Itoh et al. (2003)
Gns1	Fungal disease resistance	Nishizawa et al. (2003)
Ech42, nag70, gluc78	Fungal disease resistance	Liu et al. (2004)
OsNPR1	Bacterial disease resistance	Yuan et al. (2007)
AtNPR1	Fungal and bacterial disease resistance	Quilis et al. (2008)
Cht42	Fungal disease resistance	Shah et al. (2009)
Pi-d2	Fungal disease resistance	Chen et al. (2011)
OsMPK6	Bacterial disease resistance	Shen et al. (2010)
Xa3/Xa26	Bacterial disease resistance	Li et al. (2012)
HPL3	Bacterial disease resistance	Tong et al. (2012)
ACS2	Fungal disease resistance	Helliwell et al. (2013)
OsGA20ox3	Fungal and bacterial disease resistance	Qin et al. (2013)
RTBV coat protein	Viral disease resistance	Ganesan et al. (2009)
RSTV RNA	Viral disease resistance	Verma et al. (2012)
PINII-2X	Insect resistance	Bu et al. (2006)
ASAL	Insect resistance	Bharathi et al. (2008)
ASAL, GNA	Insect resistance	Bharathi et al. (2011)
DB1	Insect resistance	Yoshimura et al. (2012)
cry1Ab	Insect resistance	Shu et al. (2000)
cry2A	Insect resistance	Chen et al. (2005)

by the pathogen under field conditions (Tu J et al. 2000a). Wang et al. (2017) developed transgenic rice cultivar Nipponbare by the introduction of Xa10-like genes. The Xa10-like gene encodes for AvrXa10 (transcription activator-like effector) which binds to Xa-10 and activates its expression. Upon subsequent infection by *Xanthomonas oryzae* pv. *oryzae*, transgenic rice cultivar revealed improved tolerance to bacterial blight and showed higher yield.

Cao et al. (2007) reported a disease-resistant (*R*) multigene family comprising of *Xa3*/*Xa26*, *MRKa*, *MRKc* and *MRKd* encoding a leucine-rich repeat (LRR) receptor kinase-type protein in rice cultivars that govern tolerance to *Xanthomonas oryzae* pv. *oryzae*. Their results revealed few R genes under strong constitutive promoter conferred tolerance to *Xanthomonas oryzae* pv. *oryzae* as compared to their native promoters. Rice plants harbouring another gene *Xa26*, isolated from rice, also revealed high level of resistance against bacterial blight (Sun et al. 2004).

Genetically engineered rice plants expressing AP1 (ferredoxin-like protein) isolated from sweet pepper showed improved resistance to *X. oryzae* (Tang et al. 2001). This confirms the ap1 gene could be used to induce bacterial resistance in disease-susceptible rice cultivars (Table 2).

Several genes that confer resistance against several fungal diseases were identified and subsequently employed in genetic engineering programs to improve tolerance to fungal attack in disease-susceptible rice cultivars. Dai et al. (2010) have recently reported a cloned *Pi-ta* gene that confers substantial tolerance against rice blast caused by a pathogenic fungi *Magnaporthe grisea*. Liu et al. (2009) have mapped the loci that govern tolerance to sheath blight caused by a pathogenic fungi *Rhizoctonia solani*. They were successful in identifying several molecular markers associated with sheath blight resistance by means of crossing between resistant transgenic and sensitive non-transgenic rice cultivars (Table 2). Datta et al. (2003) reported a gene *PR-3* that confers tolerance to sheath blight and hence can be used in transgenics for the improvement of fungal disease resistance. Transgenic rice harbouring *Rir1b* gene (defence-related gene) isolated from cereals reflected improved resistance to rice blast (Mauch et al. 1998; Li et al. 2009). Different proteins/genes isolated from different organisms have been recognised as potential source to confer resistance against several fungi species in rice plants (Kumar et al. 2018). For instance, transgenic rice harbouring and co-expressing *ap24* (tobacco osmotin), *chi11* (rice chitinase) (Sripriya et al. 2017) and chitinase and oxalate oxidase 4 (Karmakar et al. 2016) and overexpression of *LOC_Os11g47510* chitinase gene showed more resistant to sheath blight disease (Richa et al. 2017). Several proteins such as puroindoline proteins (Krishnamurthy et al. 2001), flavonoid pathway genes (Gandikota et al. 2001), trichosanthins (Yuan et al. 2002), defensins (Kanzaki et al. 2002), phytoalexins (Hasegawa et al. 2010) and antifungal protein from *Aspergillus flavus* (Coca et al. 2004) are known to play a vital role in combating the fungal diseases and can be promising candidates in transgenics.

Transgenics have also been very successful in developing insect-resistant crops including rice (Brooks and Barfoot 2013). Fujimoto et al. (1993) have reported the development of insect-resistant rice plants about two decades ago. Transgenic rice harbouring *cry* genes isolated from *Bacillus thuringiensis* are currently under field trials, the preliminary results reveal a substantial resistance against stem borers and leaffolders (Cohen et al. 2008; Wang et al. 2014). Similarly, High et al. (2004) have reported that Bt rice showed significant resistance against lepidopterous pests in Asia. Transgenic rice carrying a synthetic c*ry1Ab* gene reflected substantial tolerance to several lepidopterous pests of rice (Shu et al. 2000). Moreover, field studies led to the identification of two lines from Bt rice plants that showed complete resistance to lepidopteran pests (Kumar et al. 2008; Wang et al. 2014). In China hybrid rice plants with improved tolerance to rice leaffolder and yellow stem borer were developed (Tu JM et al. 2000b; Chen et al. 2011). In Pakistan and Mediterranean region, insect-resistant Bt rice have been developed (Breitler et al. 2004) which reflected complete resistance against target yellow stem borer and rice leaffolder. Studies are being carried out to pyramid *cry1Ab* or *cry1Ac* with either *cry2A* or *cry9C* for high resistance in Bt rice (Alcantara et al. 2004; Ansari et al. 2015). In

addition to *cry* genes, *gna* lectin gene isolated from snowdrop (*Galanthus nivalis*) induced higher levels of tolerance against several pests (Ramesh et al. 2004). The transgenic rice harbouring protease inhibitors and lectins showed improved tolerance against insect pests, and hence they may also serve as potential source to develop rice with improved resistance against several insects (Kumar et al. 2008) (Table 2).

3 Genomics

Biotic stress incurs a substantial decrease in the average annual yield of rice in rice fields worldwide (Heinrichs and Muniappan 2017). In the current scenario of climate change and evolution of pests and pathogens, plants face biotic stress at rapid pace (Cohen and Leach 2019). Conventional breeding strategies practiced thousands of years have resulted into varieties that were much tolerant to disease outbreaks (Buddenhagen 1983). However, the co-evolution of new virulent strains on a much faster pace further posed challenges before plant breeders and geneticists. The conventional breeding approaches are cumbersome, laborious and requires a long duration of time to improve a trait and all these drawbacks have led to the rise of marker-assisted breeding for developing varieties with improved tolerance to diseases. Initially, molecular markers like RFLP, RAPD, AFLP, SSRs and SNPs have played a major role in marker-assisted breeding for developing varieties with increased resistance to a wide range of biotic stresses (Table 3). Later on mapping of quantitative trait loci provided more insights into the underlying mechanism of tolerance to viral, bacterial, fungal and insect pest attack in rice. A recently developed genome editing techniques have superseded the drawbacks of conventional breeding approaches and have paved a new way for crop improvement. Genome editing approaches have been used to modify various disease-related genes to enhance disease resistance in rice. In genome editing techniques, site-specific nucleases are employed to engineer genes of interest at desired loci in the genome. Transcription activator-like effectors (TALEs) from *Xanthomonas* species such as AvrXa7 and PthXo3 target and modify the sugar transporter SWEET gene and sucrose efflux transporter OsSWEET14 gene to facilitate the influx of sugars from the plant cell to the pathogen (Antony et al. 2010; Cohn et al. 2014) (Table 3). Transcription activator-like effector nucleases (TALEN) technology was used to modify the bacterial protein binding site on OsSWEET14 gene to impart resistance against *Xanthomonas* causing bacterial blight (Li et al. 2012). TALEN technology is effective in disrupting EBEtal7 binding site in promoter of Os09g29100 gene, which could significantly decrease bacterial blight (Cai et al. 2017). Li et al. (2012) reported that collaborative approach of targeted mutagenesis and TALEN technology was effective in disrupting the *Os11N3* gene susceptible for bacterial blight in rice. Recently, a simple robust and effective gene editing technology have been developed wherein the disease susceptible genes can be targeted and edited to improve disease resistance in rice. CRISPR/Cas9 have been used to target and edit by deleting nine and seven nucleotides from promoter of *OsSWEET14* and *OsSWEET11* genes,

Table 3 Role of genomics in improving the resistance of rice crop against biotic stresses

Gene (s)	Improved trait (s)	Reference
OsSWEET13	Enhanced resistance to bacterial blight	Li et al. (2012)
OsSWEET13	Enhanced resistance to bacterial blight	Zhou et al. (2015)
OsSWEET13	Enhanced resistance to bacterial blight	Blanvillain-Baufum et al. (2017)
Os09g29100	Enhanced resistance to bacterial leaf streak	Cai et al. (2017)
OsERF922 CRISPR/Cas9	Enhanced resistance to blast disease	Wang et al. (2016)
cry1Ab or *cry1Ac*	Yellow stem borer, stripe stem borer	Shu et al. (2000)
cry1Aa or *cry1Ab*	Stripe stem borer	Breitler et al. (2004)
cry1Ab and *cry1Ac*	Yellow stem borer	Ramesh et al. (2004)
cry1Ab	Stripe stem borer	Cotsaftis et al. (2002)
cry1Ab	Yellow stem borer, rice leaffolder	Bashir et al. (2005)
cry, *Xa21* and *RC7*	Yellow stem borer, bacterial blight, sheath blight	Datta et al. (2003)
gna and *cry1Ac*	Homopteran, coleopteran and lepidopteran insects	Nagadhara et al. (2003)
Itr1	Rice weevil	Alfonso-Rubi et al. (2003)
cry1Ac and *cry2A*	Yellow stem borer, rice leaffolder	Mahmood-ur-Rahman et al. (2007)
Bt and *CpT1*	Insect resistance	Rong et al. (2007)
Bt, protease inhibitors, enzymes, and plant lectins	Insect resistance	Deka and Barthakur 2010
cry2Aa	Insect resistance	Wang et al. (2012)
cry1Ab	Insect resistance	Wang et al. (2014)
xa5, *xa13* and *XA21*	Bacterial Blight resistance	Singh et al. (2001)
Xa39(t)	Bacterial	Sundaram et al. (2014)
Xa38, *xa13*, *XA21*	Blight resistance	Sundaram et al. (2014)
XA21, *xa13*, *xa5* and *Xa4*	Bacterial	Sundaram et al. (2014)
XA21, *xa13 and xa5*	Blight resistance	Sundaram et al. (2014)
XA21 and *xa13*	Bacterial	Sundaram et al. (2014)

thereby increasing bacterial leaf blight resistance in rice (Jiang et al. 2013). In indica rice, IR24 a null mutation in OsSWEET13 was created by means of CRISPR/Cas9 to avert its neutralisation by the TAL effector gene pthXo2, thereby increasing tolerance against bacterial blight disease (Zhou et al. 2015). Wang et al. (2016) reported the enhancement of tolerance against rice blast by targeting the *OsERF922* gene via a CRISPR/Cas9 technology. Another CRISPR/Cas9-mediated editing of eIF4G gene has led to an improvement in tolerance against rice tungro spherical virus RTSV (Macovei et al. 2018). This confirms that CRISPR/Cas9 is a coherent tool for improving resistance against almost all diseases in rice.

4 Conclusion

Biotic stresses that devastate the rice production include virus, bacteria, fungi, nematode and insect pests. Among different breeding approaches, mutagenesis have proven very effective tool for enhancing the genetic variation and improving resistance to biotic stresses. The recently developed genome editing techniques have superseded the drawbacks of conventional breeding approaches and have paved a new way for crop improvement. Genome editing approaches have been used to modify various disease-related genes to enhance disease resistance in rice. Overall, the modern biotechnological tools such as mutagenesis, transgenics and genomics have led to the identification, cloning and characterisation of genes (from different organism) followed by its insertion into the rice plants with the aim of decreasing the yield loss incurred by the different biotic stresses.

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Temporal and Spatial Dynamics of Microbial Communities in a Genetically Modified Rice Ecosystem

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Abstract

Genetically modified crops are new products of agriculture biotechnology. Rice is one of the top food crops that are modified genetically to fulfill the increasing food demand of the world. However, the benefits and risks of genetically modified crops are the topics of hot debate. Limited reports regarding the effects of genetically modified rice on the structure and function of soil microbial communities are available. Moreover, conclusions based on these studies are very perplexing and create a state of confusion regarding the impact of GM rice and their released products on soil microbiota and ecosystem. Few of the reports on transgenic rice recommend that if the transgene products from the GM rice become accumulated more than their utilization and/or biodegradation, it might cause accretion of these products in the soil beyond their safety level which may cause the long-term impact on the soil ecosystem including soil micro-fauna and micro-flora. Temporal and spatial factors are too much important in determining the influence of GM rice on communities of soil microbes and environment. Therefore, the following chapter is prepared to gather the information regarding

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the impact of GM rice and its residues on the soil microbial communities. In the first part, a brief description on the interaction of GM crops with soil microbes is presented, and in the second part studies regarding the impact of transgenic crops on the soil microbial communities are reviewed. At the end, a framework to evaluate the effect of GM rice on soil ecosystem relative to its parental variety is suggested.

Keywords

Transgenic rice · Microbial communities · *Oryza sativa* · Ecosystem · GM rice

1 Introduction

The key challenge of the twenty-first century is the provision of food security to the world's growing population and to approximately twofold more food requirement by 2050 in comparison to current food demand, a greater portion of which is expected to be fulfilled by cereals, especially rice (Long-ping 2014; Alfred et al. 2014; Rotter et al. 2015). This challenge moves the agriculture sector from the green revolution to the gene revolution via advanced biotechnology. So, after the computer and the industrial revolution, the gene revolution has been established as the 3rd technological revolution (Jazdi 2014). Agriculture gene revolution involves the modification and organizational understanding of traits inside chromosomes of a species and alteration of organism's traits via individual gene transfer from one species to another species, i.e., transgenic construction. Transgenic molecular technology permits the creation of the GM plant in which desirable traits using foreign DNA can be transferred from any source to the specific crops.

Various possibilities to improve crop efficiency are offered by transgenic technology such as to make an improvement by gene transfer which results in resistance development to pests, herbicides, diseases, and abiotic and biotic stresses and enhancement of food quality traits, i.e., postharvest storage and nutritional contents (James 2011; Lombardo et al. 2016; Wijerathna-Yapa 2017; Singh and Prajapati 2018; Samal and Rout 2018). In 1994, first transgenic tomato FlavrSavr was released by Calgene development (James 2006) which resulted in fast commercial development of herbicide-tolerant cotton, soybean, and maize in 1995 and insecticidal proteins expressing crops derived from *Bacillus thuringiensis* (Bt), a soil bacterium conferring resistance to Diptera and Lepidoptera larval pests in 1996, respectively (James 2011). An area of 189.8 million ha was reported to be under GMOs cultivation world widely in 2017 (ISAAA 2017).

In the current scenario, the sustainability of transgenic plants and true assessment of their impacts on the environment is a major challenge despite the remarkable changes in transgenic technology in agriculture. Environmental risk assessments are used to address the aboveground impacts of GM plants regarding their cultivation or release. However, the effects of components of genetically modified crops which are belowground have been neglected, although the importance of soilborne organisms along with plant dominant role with respect to energy and carbon underground inputs is well-known. Dominant key components are soil microorganisms with

respect to the soil activities and biomass. They greatly determine the terrestrial ecosystem and account for >80% of the total biomass. In addition, soilborne microorganisms' direct and indirect interactions with plants create sound feedback which influences the dynamics of vegetations (Faucon et al. 2017).

In several countries, the commercialization of the GM rice is extremely controversial due to their harmful effects on the human health, soil ecosystem, and environment. Major issues include unintentional horizontal gene flow from transgenic crop residues to native microorganism and plants, possible development of invasive species of plants, induction of resistance in pests, and impacts of transgenic crops on the non-target soil life especially beneficial soil microorganisms (Wolfenbarger and Phifer 2000; Liu et al. 2018, 2019; Li et al. 2019). On non-target soil microorganisms, effect of transgenic crops is dependent on exposure extent and nature of recombinant protein (activity spectrum). Root exudate of insect-resistant GM plants alters the environment of rhizosphere which ultimately enhances microorganism growth in rhizosphere (Li et al. 2018; McGregor and Turner 2000; Ibarra et al. 2019).

In evaluation regarding the effect of transgenic plants on soil microorganisms, major problem is absence of an approach which must be universally accepted to make transgenic plants impact assessment on ecosystem of soil and insufficiency of baseline information on diverse agroecosystems in comparison to agroecosystems in which introduction of transgenic crops has been carried out (Bruinsma et al. 2002; Dale et al. 2002). Impacts of GM crops on soil ecosystem functioning are major concerns despite enough potential of genetic engineering technology in improving agricultural yields.

These impacts may be:

1. Direct (such as toxicity of a specific introduced gene on non-target specie of an essential function group)
2. Indirect (e.g., impacts through the unintended variations in the plant metabolism and thereby affecting the compositions and fluxes of root exudates)
3. Caused by management regime changes that used with transgenic (GM) crops (Birch et al. 2007)

Consequently, literature review is important for understanding the possible risks of transgenic plants and their observed products on diversity of soil microorganisms up till now as well as to propose a framework based on a perspective which worldwide should be followed for transgenic crop impact assessment at different regulatory stages on soil ecosystem before their release and after their commercialization.

The development of transgenic crops has brought enormous economic benefits along with ecological safety concerns, i.e., possible effects on soil microorganisms. Several studies have been carried out for investigating the effects of the transgenic crop on soil microbial communities (Garbeva et al. 2008; Lucas et al. 2013; Hunting 2013; Li et al. 2018, 2019; Liu et al. 2018, 2019; Ibarra et al. 2019). Most of the results showed that transgenic crops in comparison with conventional crops have not caused significant impacts on soil microorganisms (Blackwood and Buyer 2004;

Miethling-Graff et al. 2010; Fang et al. 2012; Chun et al. 2012; Hu et al. 2013; Singh et al. 2013; Yasin et al. 2016; Li et al. 2018; Ibarra et al. 2019). Soils are the host of a diverse life range, and although the diversity of microorganisms is not well classified, their compositions are dominant (bacteria, nematodes, fungi, and protists). Between the microbiota, communications occur in soil matrix. Even microscale changes in this considered environment can mean various changes in key factors' bioavailability such as oxygen, nutrients, and water which confers the pressure of selection on the sustainability of different microorganisms. This heterogeneity explains the soils as the most diverse habitats on the Earth planet and explains that more species are not excluded in the competition (Wardle 2002). In the cohesiveness stability of soil, the activity of microorganisms plays an important role, and most of the soil microorganisms are concentrated in the rhizospheric soils of plants (Gupta et al. 2000; Haldar and Sengupta 2015; Ali et al. 2019; Ijaz et al. 2019). So, any change which impacts the rhizosphere of plant has the potential to change the communities of soil microorganisms and activities which can be beneficial or harmful. According to multiple studies, it has been found that different plant species favor microbial communities. Root exudates of plants vary in their composition which affect microbial abundance in the rhizosphere of roots (Rehman et al. 2019; Ali et al. 2019; Somers et al. 2004). Transgenic crops change root exudates composition via introduction of a functional gene in plants. Therefore, short-term and long-term effects on ecosystem of soil should be recorded to monitor their safety regarding agriculture environment (Gupta et al. 2000). In the past, many studies have reported physiological and metabolic changes in transgenic crops. Soil microbial diversity gets influenced by products released by these transgenic crops in soil ecosystem (Sanchis 2011; Sanahuja et al. 2011; Hannula et al. 2014; Li et al. 2018; Liu et al. 2019). The effect of transgenic plants on microbial communities of soil either is negligible or has some temporal and spatial differences and can be compared with natural factors (Becker et al. 2008; Yasin et al. 2016; Li et al. 2018; Ibarra et al. 2019). Most studies on the transgenic crops with associated microorganisms have focused on the communities of bacteria, and less attention has been given to the interactions of GM crops with fungal communities. Both bacteria and fungi are abundant in the soil ecosystem and metabolically diverse and influence the soil nutrients cycles profoundly (Brodie et al. 2003; Viebahn et al. 2005; Vujanovic et al. 2007; Rehman et al. 2019). One potentially adverse environmental impact of transgenic crops on soil microorganisms is the non-target effect and variation in the microbe-mediated functions and processes in soil which could be influenced by the insect-resistant Bt crops in soils via the Bt crop cultivation and insecticidal Cry proteins (Saxena and Stotzky 2001; Griffiths et al. 2005; Dastan et al. 2019; Li et al. 2019). The ecological stability of GM crops may depend on their dynamic relationships with communities of soil microorganisms. The success of GM plants highlights the progress of the researchers in nature transformation; however, the environmental safety of GM plants has been acquiring increased attention because of larger cultivation areas and increased commercial applications (Andow and Zwahlen, 2006). Many researchers have assessed the ecological risks of GM plants since their commercialization (Velkov et al. 2005; Icoz and Stotzky 2008; Kos et al.

2009; Liu 2010; Domingo and Bordonaba 2011; Sanvido et al. 2012; Camastra et al. 2015; Leclerc et al. 2018; Schiemann et al. 2019).

Historically in many countries, rice (*Oryza sativa* L.) has been served as a principal food source, and even today it continues to do so especially in Asia (Bajaj and Mohanty 2005; Bandumula 2018). Rice was one of the first cultivated transgenic crops and was targeted for development as a GM crop (Bajaj and Mohanty 2005; Kathuria et al. 2007). On soil-dwelling biota, the effects of GM rice are exposed barely (Kathuria et al. 2007; Kim et al. 2008), and such knowledge is necessary for the verification of GM varieties. Genetic engineering of plants has been used for the improvement of crop production qualitatively and quantitatively in a cost-effective way, e.g., by introducing herbicide tolerance or by enhancing resistance against diseases and pests (Wolfenbarger and Phifer 2000; Parisi et al. 2016; Biden et al. 2018). Hence possible side effects of GM plants on the structure and function of soil microbial communities should be considered firstly. In the rhizosphere, fungi along with bacteria are very important to the functioning of soil-plant systems, and these functions range from plant pathogens and symbiotic arbuscular mycorrhizal fungi to decomposers (Carlile et al. 2001; Buée et al. 2009; Ali et al. 2019; Rehman et al. 2019). Soil ecosystems are extreme in their heterogeneity and complexity, and scientists are still unable in the identification of microorganism's vast majority in the soil system. However, there is the possibility to examine the responses of belowground microbes to transgenic plants with the introduction of molecular biological techniques and many studies in past decade endeavored to examine possible impacts of many GM crops on (Stephen and Kowalchuk 2003; Zhang et al. 2016; Verma et al. 2018; Lu et al. 2018; Manici et al. 2018; Bai et al. 2019). Belowground components and GMPs interactions can help in transgenic technology reviving for future transgenic traits development keeping in mind the environmental and food safety.

2 Interaction Between Transgenic Plants and Soil Microorganisms

Soils are the richest habitats of life forms on the planet Earth and include bacteria, fungi, viruses, protozoa, lichens, algae, as well as vertebrates, micro-, meso-, and macrofauna (Fig. 1)

In the maintenance of resilience, soil microbial diversity plays an important role (Mikola et al. 2002; Helfrich et al. 2015; Jacoby et al. 2017). Microorganisms and plants are influenced to a greater extent by the biological and physiochemical properties of soil and vice versa. Plants influence the soil via nutrients and gas-water exchange and rhizodeposition of plant litters. Root exudates, i.e., pH, organic acids, sugars, ions, and phosphatases, promote mineral solubilization and desorption and alter the rhizospheric activities (Cardon et al. 2001; Sharma et al. 2013; Koranda et al. 2011; Brzostek et al. 2013; Guyonnet et al. 2017).

The effect of transgenic crops on ecosystem of soil is a serious issue, and there must be a clear difference between impact on soil microorganism diversity and soil

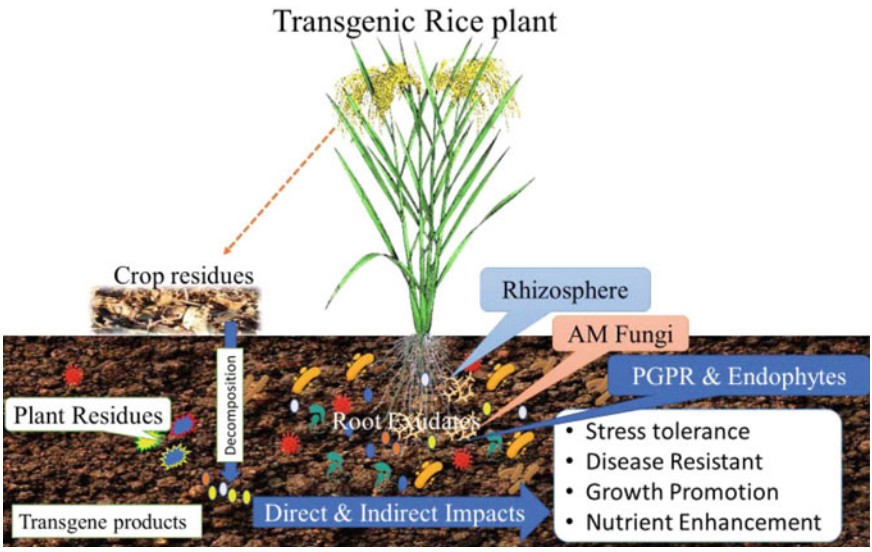

Fig. 1 Transgenic rice plant and microorganisms

function. A reduction in microbial diversity is not necessarily to cause reduction in function of soil. Several studies have been conducted to investigate interactions between diversity and function. According to them functions are maintained by a small number of species (Tesfaye et al. 2003; Nielsen et al. 2011). According to researchers, there is no significant relation between diversity and function, and species richness is also not significant in functioning of soil (Dassen et al. 2017; Sasaki and Lauenroth 2011; Bardgett 2002; Seymour et al. 2015). Soil and plants are main drivers for soil ecosystem that provide a heterogenous environment both spatially and temporally to plants and soil microorganisms.

2.1 Natural Interactions

Soil microorganisms naturally interact with the roots of plants within the rhizosphere and on the rhizoplane (Glick 2015; Ali et al. 2019; Ijaz et al. 2019). Through the rhizospheric effect, plant roots have a direct impact on the density and composition of soil microorganisms in the rhizosphere. The microbial population normally is more active and larger in the rhizospheric zone in comparison to bulk soil. In the rhizosphere, chemical and physical conditions are different, and plant-roots provide most of the substrates for energy and biosynthesis by microorganisms in the soil. Plant roots secrete organic acids, amino acids, carbohydrates, lipids, vitamins, hormones, enzymes, lysates, mucigel, and mucilages root exudates into the rhizosphere (Shukla et al. 2011; Vranova et al. 2013; Glick 2015; Ali et al. 2019). Therefore, plants through their rhizosphere have significant effects on soilborne microbial processes and communities, and soil microorganisms vice versa affect

the plant performance and growth through their activities. According to the introduced transgenic traits, transgenic plants naturally at different growth stages excrete different transgenic products (e.g., T4 lysozyme and Bt toxins) actively into soil ecosystem through the different routes such as root exudates from roots, from plant injuries via leachates, from root or root cap cells sloughing off, through the senescent leaves decomposition, and in the field remaining transgenic plants biomass after the final harvest (Conner et al. 2003; Liu 2009; Wang et al. 2013a). It has been reported that Bt endotoxin released from root exudates, pollen, and crop residues rapidly binds with kaolinite clay minerals, montmorillonite, organo-mineral complexes, and humic acids (Saxena and Stotzky 2001; Crecchio and Stotzky 2001; Helassa et al. 2013; Liu et al. 2019) studied the fate of Cry protein released from transgenic rice in different four types of paddy soil and concluded that Cry proteins could be adsorbed in paddy soils, among which clayey soil give the stronger affinity. Due to this strong binding, Cry protein could not be degraded by soil microbes and thus accumulates and persists in the soil ecosystem, effecting the soil microbial communities.

2.2 Influence of Anthropogenic Activities

Anthropogenic activities also influence the interaction between soil biota and transgenic plants. These anthropogenic activities are the agricultural practices managed by humans such as irrigation applications, tillage activities, and application of several herbicides, pesticides, etc. Through the tillage practices, residues of transgenic crops are incorporated in the soil which communicates with soil biodiversity during their biodegradation and affects it either negatively or positively. On the soil surface, crop residues are left concentrated in zero-tillage practice which limits the soil microbes to come in interaction with soil surface protein, whereas plant litter in convention tillage incorporated into the soil which dilutes the concentration of transgene product but increased the number of exposed organisms (Mina et al. 2008; Axelsson et al. 2011). Zwahlen et al. (2003) conducted the field trial and observed the variations in the Bt toxin long-term persistence from transgenic corn with respect to tillage practices.

Presence of transgenic product in soil may affect the functions and structures of soil microorganisms and their community by arresting or selectively stimulating the growth of particular organism that is using the transgenic product (McGregor and Turner 2000; Li et al. 2018; Ibarra et al. 2019).

3 Effects of Transgenic Plants on the Structure and Functions of Soil Microorganisms

In the soil ecosystem, transgenic crops and release of their novel product may change the soil microbial structure either directly or indirectly. Most of the risk assessment studies on transgenic plants addressed the commercialized impacts of transgenic

plants on soil microbial community structural alteration in comparison with non-transgenic isoline.

Faragová et al. (2011) reported that soils from the transgenic alfalfa field (herbicide-resistant) showed higher significant population levels of spore-forming aerobes, culturable and cellulose utilizing bacteria in comparison with non-transgenic parental lines. Saxena and Stotzky (2001) observed that there is no apparent effects of corn derived Bt toxin on the populations of soil bacteria, fungi, protozoa and earthworm. Liu et al. (2008) reported that the Bt rice (KMD1) which expressed the Cry1AB have no significant adverse impacts on fungal and bacterial community or their essential processes. Variations in rhizosphere-associated soil microbial communities outweigh the triazophos application and modifications of Cry1AB over the 2 years of rice cropping. Wei et al. (2012) reported lesser impacts on the rhizosphere-associated actinomycetes and fungal and bacterial communities. Li et al. (2018) in a study on interaction of Bt cotton and microbial community observed the varied response of some dominating and rare genera of bacteria to the growing of Bt cotton. Further, different root zones of Bt cotton harbored diverse bacteria and different community structures, and when compared with traditional cotton varieties, nearly similar bacterial community composition was found in the rhizosphere of Bt cotton varieties. Liu et al. (2019) studied the impact of degraded straw of transgenic rice on microbial communities of two different paddy fields, and observations show that the total viable microbial cells, bacteria, and archaea were more in number in fields having transgenic rice residues, but the difference of community composition of Bt and non-Bt rice was non-significant.

3.1 Reasons for Soil Microbial Responses to Transgenic Plants

Several studies reported the response of soil microbial variations with respect to transgenic plant environments and varieties conditions. Studies showed no or minor Bt genes expressing particular impacts on soil microbial community, and dominance of microbial community can be determined by type and age of plants and by other environmental factors such as soil pH, soil texture, redox potential, moisture, N-concentration, precipitation, temperature, etc. (Blackwood and Buyer 2004; Baumgarte and Tebbe 2005; Icoz et al. 2008; Chen et al. 2011; Wei et al. 2012; Li et al. 2018; Ibarra et al. 2019; Liu et al. 2019). Oger et al. (2000) reported that the changes induced by transgenic plant in soil microbial communities remain persistent for a longer time. Heuer et al. (2002) reported that transgenic potato DL4 line-associated rhizospheric community structure varies from the control (DES) and DL5 transgenic line-associated community structure. Head et al. (2002) reported that from the cultivated Bt cotton fields soil samples, Cry1Ac proteins remain undetectable and due to postharvest tillage have incorporated the residues of Bt cotton plants. The intensity and persistence of the impacts of transgenic plants on microbial community structure depend upon environmental conditions of the cultivation site (Dunfield and Germida 2001, 2003). Christopher and Jeffrey (2004) for bacterial and fungal CLPP and PLFA observed the Bt corn bulk and rhizospheric soil samples and

found differences in the bulk and rhizospheric soil microbial community of 6.3−3.8 and 73%, respectively. Liu et al. (2008) reported that under laboratory and field conditions, enzymatic activities did not differ significantly in the rhizosphere of nonparental rice and transgenic Bt rice and indicated that the effects of the genetic modifications could have masked by the crop growth effects. Flieβbach et al. (2012) reported that under field conditions soil dehydrogenase activity reduces Bt maize verities in comparison to non-Bt counterpart and suggested that soil-mediated processes can modify due to transformation plant composition anticipated changes. Few reports mentioned the flow of rice Bt protein into the rhizosphere during rice growth, but the distribution of the protein was in a small area around the plant roots (Wang et al. 2013a, 2013b), while some studies confirm no protein contents in the soil (Wang et al. 2006). Non-significant difference in the concentration of Cry1AB/1AC protein in Bt rice and non-Bt rice was explained by Wang et al. (2019) as a very low concentration of Cry protein was measured in soils sampled from the transgenic rice fields; however a nearly equal concentration of this protein also is observed in non-Bt rice soil that may be because of the possible presence of *B. thuringiensis* in the indigenous micro-flora (Wang et al. 2013a). Different factors such as soil temperature, soil pH, moisture content, and aeration of the soil affect the decomposition of Cry protein (Wang et al. 2007; Feng et al. 2011), and its decomposition under alkaline submerged conditions becomes relatively difficult (Wang et al. 2007). Wang et al. (2019) recorded a very slight impact of transgenic rice on the composition of the soil bacteria; however significant variance between Bt and non-Bt rice treatments was noted. Likely, several studies have been conducted to measure the impact of transgenic rice on soil bacterial community composition, and results were non-significant when compared between transgenic and non-transgenic lines (Chun et al. 2012; Liu et al. 2008; Lu et al. 2010). Impact of rice residues, obtained from Bt-transgenic rice and protoporphyrin oxidase-transgenic rice, on soil bacterial and fungal community composition was investigated (Lu et al. 2010; Chun et al. 2012), and results showed the no significant difference between the transgenic line and their respective parent variety. A soil, cultivated with Bt transgenic rice up to 8 years, gave non-consistently significant alteration in the soil microbial biomass and enzymatic activities (Zhaolei et al. 2017). In another observation, direct application of Cry1Ac protein rendered no significant change in indices of soil microbial diversity and similarity (Zhaolei et al. 2018). Based on these results, it can be suggested that soil microbial community composition does not change significantly in soils cultivated with transgenic rice. However, rhizospheric microbial community composition, generally, are strongly influenced by root exudates of transgenic rice (Yang and Crowley 2000; Breidenbach et al. 2017; Li et al. 2018; Long et al. 2018). In this context, Dennis et al. (2010), who were analyzing the spatiotemporal constrictions of root exudates and ubiquitous nature of the principal compounds present in root exudates, suggested to take into account the inclusive involvement of carbon pools from other rhizosphere for close estimation of impact of rhizodeposits on soil microbiome. Hence, it was possible a paddy soil with the same agronomic practices rendered slight or no significant variation in microbial community composition in the same soil when cultivated with transgenic and parental rice varieties. This

suggests a very low impact of transgenic rice on soil microbiome in the rhizosphere. In conclusion, rice residues did not show a significant variation between the parental variety and its transgenic plants. In spite of the detection of low concentration of the Bt protein in soil cultivated with Bt transgenic rice, observations of the high-throughput sequencing exhibited that composition of rhizospheric bacterial community was not affected by growing the Bt transgenic rice compared with the parental variety (Wang et al. 2019).

3.2 Spatiotemporal Impact of Transgenic Plants on Soil Ecosystem

There is a significant role of temporal and spatial factors of specific cultivation site in determining impact of transgenic plants on soil ecosystem. All changes that take place in soil by introducing transgenic plants need to be verified through replication of experiment for a longer duration. To understand transgenic crops' impact on soil ecosystem, future research must be focused on inquiring non-targeted characters of transgenic crops. Different mechanisms which affect biological processes including root exudates composition and structure of soil must be studied. Major functions performed by soil microorganisms are nutrient cycling and energy flow. Energy flow occurs from primary producers to consumers (primary, secondary, and tertiary) and from plants to microbes (decomposers and saprophytes). Any negative or positive alteration in composition and structure of soil microorganisms due to transgenic plants will affect energy flow. This flow takes place through food chains as well as food webs on larger scale in an ecosystem. Any impact of these transgenic crops on food web will have a significant effect on energy flow. There are no enough reports available about soil ecosystem-energy dynamics with respect to transgenic crops. Future research must be focused on soil ecosystem and transgenic plant to fill existing gap. Plant nutrients' major pool is soil organic matter, composed of dead and decomposed animal and plant residues. There are various reasons of nutrient return variations from transgenic crops residues in comparison to non-transgenic. These include:

1. Changes in composition, physical form, and quantity of residue produced from transgenic plants.
2. Soil microorganisms which play an important role in nutrient transformations either inhibit or stimulate them due to presence of chemical compounds in transgenic crop residues.
3. Management practices such as tillage may affect abiotic and biotic factors which are involved in crop residue decomposition and nutrients turnover. Changes in crop residue composition take place by introduction of transgenic characteristic, such as in Bt crops.

Bt rice cultivars were having high efficacy against pests of lepidopteran and were also expressing various cry genes (Wang et al. 2010; Han et al. 2007; Ye et al. 2003). A change in lignin composition was observed by regulation of coniferaldehyde 5-hydroxylase encoding gene in rice (Takeda et al. 2017). Under temperate and tropical conditions, composition of crop residues and importance of indices regarding residue quality was examined. Parameters of residue quality such as lignin/N ration, N initial content, soluble carbon concentration, C/N ratio, polyphenol content, and polyphenol/N ratio were studied to find out effect on N mineralization and decomposition (Fernandes et al. 1997). Plant residues' decomposition rate slows down when polyphenol content or lignin/N increases which ultimately reduces nitrogen availability for short term (Fernandes et al. 1997). So, decomposition rate of plant residues and organic nitrogen-mineralization gets affected by lignin contents of Bt crops. This reduction in nitrogen-mineralization rate because of transgenic residues may affect nitrogen cycle in soil ecosystem. Structure of microbial community and their functioning is determined by crop residues which are primary carbon source for soil microbes (Icoz and Stotzky 2008).

Microbes involved in degradation of crop residues are considered of great importance for biological functions regulation in soil ecosystem. While effects due to genetic modification in plants related to residue decomposition of Bt rice are less, however, there is a significant influence of Bt plant on microbial composition of soil (Castaldini et al. 2005). Incorporation of crop residues in soil increases contact of soil microbes with soil (Ali et al. 2019; Lachnicht et al. 2004). According to several studies related to abundance and spatial distribution of microbial groups, a prevalence of microbial communities between oxygen-depleted layer and surface layer of wetland soils has been found (Noll et al. 2005; Lüdemann et al. 2000). To find out impact of tillage practices with respect to transgenic crops and their impact on soil ecosystem is very important. A decay of Bt transgenic cotton and roundup ready cotton residues was observed under two systems such as no-tillage and conventional tillage (Lachnicht et al. 2004). Mass loss in subsurface decomposition was almost 54% in conventional tillage. While in surface decomposition of transgenic residue of cotton in no-tillage practice, it was only 26%. The reason was greater contact of transgenic biomass with microorganisms under conventional tillage practice which ultimately affected their function and structure. No tillage practices reduced chances of erosion which ultimately increases transgenic crop biomass deposition on soil surface. Moreover, an improvement in weed control with crop tolerance to herbicides has encouraged adoption of conservation tillage because of more crop residues on soil surface and less erosion (Busari et al. 2015; National Research Council 2002; Blevins et al. 2018).

Any change in activity of enzymes affects normal functioning of soil microbes, and this change occurs due to specific chemical compounds of transgenic residues. Enzymatic activities such as dehydrogenase, alkaline phosphatase, protease, and cellulase were compared in soils amended with non-transgenic and Bt transgenic straw of rice crop (Wu et al. 2002). According to results, soils having transgenic rice straw amendment were exhibiting high activity of dehydrogenase than control, while dehydrogenase and alkaline phosphatase activities were lower in rhizosphere of

transgenic alfalfa (Donegan et al. 1999) than non-transgenic isoline. Plant nutrition and phosphorous mineralization is performed by phosphatase, while organic compounds' biological oxidation is a function of dehydrogenase enzyme which is also involved in exhibiting total accountable microbial population for organic matter decay (Frankenberger and Dick 1983). Activities of soil enzyme in response to transgenic crop may depend on the concentration of Bt protein and the quantity and quality of enzymatic substrates. Firstly, concentration of Bt protein and substrates are associated with the quantity of crop residues present in the soil which is increased upon incorporation of residues on soil surface. This concludes that crop residues incorporation will results in different enzymatic activities. Secondly, amount of Bt protein with the crop type grown on a specific soil, for example, Bt protein released by rice plant is up to 1.5 ng g^{-1} dry soil which is too much lower than other crops (e.g., 56 ng g^{-1} dry soil; Yang et al. 2012). Thirdly, growth stage of the plant also determines the release of Bt protein, i.e., higher amounts during middle growth stage than early growth stage (Olsen et al. 2005; Xiao 2013). So, during middle growth stage, rice plant will relatively release more substrate, and there will be different response from soil enzyme during different growth stages of plant (Aulakh et al. 2001). Soil enzyme activity alterations indicated that transgenic plants have an indirect impact on the cycling of nutrients in the soil. Studies are very limited on the response of soil microbe's to transgenic plants under sustainable and conventional agricultural practices. Although transgenic Bt crops are engineered to express δ-endotoxin proteins in almost all parts of the plant, variation in the amount of insecticidal proteins occurs according to the age of the plant (Dong and Li 2007; Llewellyn et al. 2007; Poongothai et al. 2010) and with respect to different plant parts (Adamczyk et al. 2001; Wu et al. 2002; Badea et al. 2010). Temporarily, the variation in efficacy of Bt crops happens logically because of the fluctuation of δ-endotoxin proteins (Gore et al. 2001; Wan et al. 2005; Kranthi et al. 2005; Siebert et al. 2009; Khan et al. 2018).

Normally, during the early stages of microbial growth, the levels of insecticidal protein and efficacy were high and then further declined (Kranthi et al. 2005; Olsen et al. 2005). Temporal or spatial variation in efficacy may amplify the possibility of existing pests. In this way, it has become a major concern of Bt transgenic researchers, crop growers, and breeders (Oosterhuis and Brown 2004; Khan et al. 2018). Although numerous researches have been focused on the temporal and spatial expression of Bt genes and their efficacy in transgenic crops, much less concern was paid to the physiological processes behind it and the ways to overcome it by crop management practices in the field. Improved remobilization of the soluble protein decreases the efficacy against target pests in late season (Pettigrew and Adamczyk 2006). Nevertheless, other researches established that there was no association between them is present and argued that other physiological mechanisms for the modifications of survival of target pests in Bt-transgenic plants have existed (Gore et al. 2001; Olsen et al. 2005). Consequently, agronomists and physiologists should pay more consideration to study the effect of physiological mechanisms on the variation of Bt gene expression. Crop management practices are also needed to explore the potential of Bt transgenic crops with both high grain yield and efficacy against target insects. Transgenic crops such as herbicide-tolerant crops could

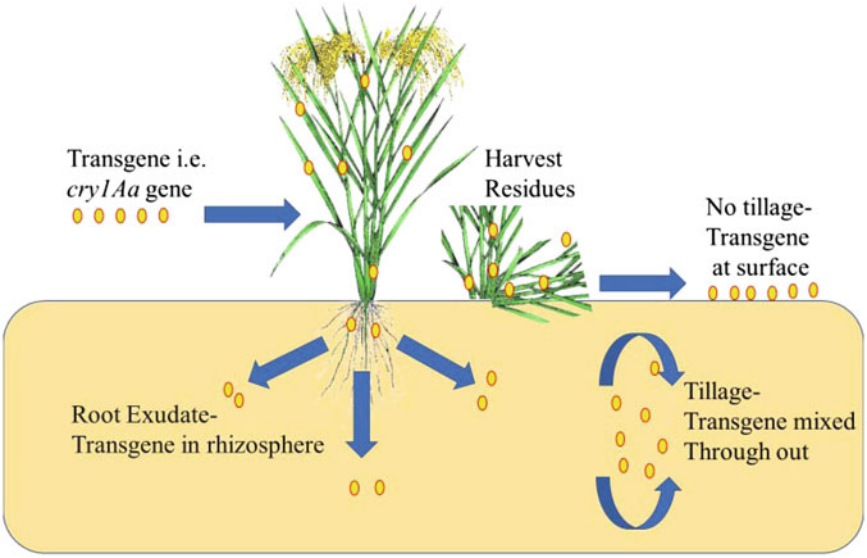

Fig. 2 Effect of tillage practices on distribution of transgene from transgenic crop

support a decline in tillage by the mechanism of direct drilling into the weedy field, which is proved to be beneficial to soil organisms. More detailed experiments are required in the future to recognize the impact of various agriculture practices of transgenic plants on the functions of soil microbes.

Rhizosphere of the transgenic plant is considered as the hot spot of gene transfer due to the possibilities of horizontal gene transfer between the transgenic plant and other soil organisms (Timms-Wilson et al. 1999) (Fig. 2).

Structural and functional variations due to the belowground gene exchange between the soil microbes and transgenic plants has been predicted (Barton and Dracup 2000; Eastham and Sweet 2002; Mina et al. 2008). The plant roots exudate DNA, and in this way, DNA becomes available to soil microorganisms. The persistence of plants DNA in the soil depends on several abiotic and biotic factors, which include the presence of DNAse enzymes and contents and type of clay minerals in the soil (Blum et al. 1997; Widmer et al. 1997; Nielsen et al. 2007; Gardner and Gunsch 2017). During natural transformation, free DNA molecules are released in the soil as a result of plant decomposition and piled on the surface of soil particles. However, for natural transformation to occur, a potential active bacteria along with free DNA must be present very closely (Smalla et al. 2000; Nielsen et al. 2007; Gardner and Gunsch 2017). Natural transformation is a method that will allow the spread of foreign transgenes, such as antibiotic-resistant markers, to inhabitant soil bacteria (Neal et al. 1973; Page et al. 1998; Nielsen and Van Elsas 2001; Pedersen et al. 2015). Transfer of antibiotic-resistant genes to the pathogenic microbes that are present in the soil is the major subject of concern from gene exchange. This will make them resistant to treatment with these types of antibiotics.

Uptake of *Bt* toxin genes by soil microbes is considered another possible complication that might occur in soil. This may lead to more *Bt* toxin assembly in the soil, the extent of which may be destructive to soil microbes that ultimately contribute to soil fertility. Meanwhile, changes or disturbances in the functioning of soil microbes occur due to horizontal transfer of genes among soil microbes and transgenic plants.

4 Mechanisms Underlying the Impacts of Transgenic Plants on Soil Microorganisms

Foreign genes and transgene products are two major forms of residues of transgenic crops that can affect soil microbial communities discussed as follows.

4.1 Foreign Gene Products

Soil microbial populations are affected by the release of transgenic protein from the transgenic crops through the root exudations (Giovannetti et al. 2005; Helassa et al. 2013; Liu et al. 2018, 2019). Transgene in the GM crops amenably affects the soil microbial communities through the activities of transgenic proteins and promoters and hence results in the variation of specific groups of microbes. Model expression of crystal proteins in plants for insect resistance is their accumulation as well as persistence in the soil. Binding of Bt protein to the soil clay and humic acid has been confirmed by Zwahlen et al. (2003). This bound protein has ability of retention and ultimately the insecticidal potential up to 235 days and cannot be easily decomposed by soil microbes. This action of Bt toxin is retained and occasionally improved by adsorption and binding to clay (Tapp and Stotzky 1995; Deng et al. 2019). Minute amounts of Bt toxin are similar to commercial microbial Bt formulations that are produced in the GM plants carried on in the soil for numerous weeks or months (Strain and Lydy 2015). Constant low levels of Bt toxin can influence some non-target organisms, and frequent use of Bt producing plants may cause a toxin buildup in the soil (Palm et al. 1996; Leclerc et al. 2018).

4.2 Horizontal Gene Transfer (HGT)

Foreign genes from the transgenic plants can be transferred into the genomes of native microbes present in the soil, which causes the variations in genetic traits and functional properties of these microorganisms. Probability of this genetic alteration from GM plant to soil microorganism is contingent on the existence of the homologous sequences for recombination and copy number of transgene (Demanèche et al. 2011). Soil is an active reserve of extracellular DNA. Under stressed conditions, some soil microorganisms can acquire necessary foreign DNA. When this foreign DNA enters the soil gene reserve, it becomes the part of ongoing gene transfer chain (de Vries and Wackernagel 2005; Brigulla and Wackernagel 2010). It has been

debated that 35S promoter of cauliflower mosaic virus, a part of the transgene creation present in more than 80 percent of the transgene plant, may result in environmental hazards if the horizontal gene transfer happens (Hull et al. 2000; Lee et al. 2010). However, reports by Kim et al. (2010) contradict the horizontal gene transfer, and observations show that gene transfer does not occur between the soil microorganism and transgene potato in potato fields. Observations made by the Lee et al. (2011, 2015) also support the no horizontal gene transfer from the transgenic *Zoysia* grasses to soil microorganisms. Demanèche et al. (2008) used the method of sensitivity-based hybridization method to study the transfer of blaTEM116 gene, an antibiotic-resistant gene from GM Bt 176 maize plant into indigenous soil microorganism and found no molecular or cellular proof of gene transfer into soil bacteria. It has been explained that frequency of the horizontal gene transfer from the transgenic crops to soil microorganism is far less than the natural transformation occurred in the soil. Moreover, the extent of harmful outcomes of HGT from the transgenic crops on the health of humans, animals, and soil is not projected as large as suspected (Keese 2008; Brigulla and Wackernagel 2010).

5 Impacts of GM Rice Cultivation on Soil Microbial Communities

Soil microbial communities impact the health and productivity of plants by interacting them in a several ways. Generally, plant nourishes the soil microorganisms by supplying them easily degradable substrates through excretion of root exudates (Bakker et al. 2015). In return, soil microorganisms provide the essential nutrients to the plants and improve the structure, health, and fertility of the soil by mineralizing the organic matter in soil and maintaining the C and N cycles (Lavecchia et al. 2015).

Positive interaction between the soil microorganisms and soil is crucial for sustainable agriculture because tenfold more microbial population is retained by rhizospheric soil than the bulk soil (Bardgett et al. 1999; Ali et al. 2019). This high population increases the chances of HGT in the rhizosphere of the transgenic plant which raises the concerns of transgenic crop cultivation due to their harmful effect on non-target soil microorganisms dominating in the rhizospheric soil (Palm et al. 1996; Wolfenbarger and Phifer 2000; Leclerc et al. 2018). Transgenic crops can impact the soil microbial population directly or indirectly (Liu et al. 2005). Direct impacts consist of the number of proteins produced by the GM crops and their potential action on the soil environment, while the indirect impacts consist of the variations in plant proteins and root exudates due to the alteration in the metabolic pathways that is caused by the transformation in plant genomes. Such kinds of the effects are more complicated to estimate due the diverse nature of the factors affecting the production of root exudate and community composition of soil microorganisms. Therefore, estimation of indirect impacts of the transgenic crops on soil microbial communities cannot be made using a single assay, but various assays must be used for comprehensive analysis.

Results based on cultivation methods and molecular approaches show the statistically significant, slight, or no effect of transgenic crops on soil microbial populations (Icoz and Stotzky 2008; Hunting 2013; Li et al. 2018; Ibarra et al. 2019; Liu et al. 2019). Nowadays, modern next-generation sequencing techniques such as alumina based or pyrosequencing have successfully filled the gab due to the restricted efficiencies of the previously used ecological approaches.

Several researchers have observed the varying impacts of GM rice on soil microorganisms (Saxena and Stotzky 2001; Zwahlen et al. 2003; Singh et al. 2013; Chen et al. 2017; Shu et al. 2017). For example, considerable variation in soil microbial populations due to the hazardous impacts of Cry protein released from the roots of transgenic crop and their residues biodegradation has been reported (Saxena and Stotzky 2001; Zwahlen et al. 2003; Liu et al. 2016). Many studies on the variation of soil microbial population have exposed the negative impacts of transgenic crops including rice on soil available nitrogen, phosphorus, or potassium and content of soil organic carbon (Singh et al. 2013; Zhu et al. 2014; Shu et al. 2017). Kim et al. (2014) found that a gene *CaMSRB2* mined from a pepper proved to create resistance in plants against toxic reactive oxygen species produced in response to biotic and abiotic stresses. Under stressed conditions, ROS is produced that imparts changes in the structure of proteins by oxidizing the S-bonds of methionine and cysteine molecules. Similarly, expression of the *CaMSRB2* gene in transgenic rice creates resistance against plant pathogens which further needs to be explored for other biotic and abiotic stresses (Kim et al. 2014). In case of drought stress, Kim et al. (2014) successfully induce drought tolerance in rice by expressing the *CaMSRB2* gene. However, before commercialization of drought-tolerant GM rice, a comprehensive evaluation of the environmental impact taking in consideration of the response of soil microbial community should be undertaken.

6 Impact of GM Rice-Straw Decomposition on Soil Microorganisms

There are two ways by which transgenic rice can influence the soil microorganisms (Singh and Dubey 2016): first, transgenic crops which influence through root exudates that release Cry protein and, second, decomposition of plant residues which is a major source of carbon for soil microorganism. Collective influences of these two ways determine the type of interaction between microbes and plant and structure and function of the soil microbes. Cry protein is also released during the decomposition of transgenic rice straw (Icoz and Stotzky 2008; Singh and Dubey 2016). A lot of experiments have been conducted to study the effects of transgenic crops on soil ecosystems, but very little attempts have been made to measure the influence of transgenic rice straw decomposition on soil microbial community composition. Wu et al. (2002) recorded a significant alteration in culturable bacteria, fungi, actinomycetes, and denitrifying bacteria, in rice fields amended with transgenic rice straw and non-transgenic rice straw. Similarly, Lu et al. (2010 found a statistically significant difference between the community composition of fungi at

early stages of decomposition of Bt rice residues and non-Bt rice residues in the paddy field. However, cultivation of Bt rice has only negligible (Lu et al. 2010; Wei et al. 2012) or no impacts (Liu et al. 2008; Song et al. 2014) on soil microbial community. Transgenic rice straw decomposition had major effects on soil microbial communities and credited to many factors such as the Cry protein level and common characteristics, which are determined by plant age and plant types. In addition, other environmental factors, such as temperature, pH, soil types, and water content, principally determined the microbial community (Tan et al. 2010; Wang et al. 2019).

Transgenic plants have altered genome that can result in alteration in the composition of residues of plant and later affects the decomposition of organic matter and ultimately soil nutrient cycles (Castaldini et al. 2005; Flores et al. 2005; Poerschmann et al. 2008). But some reports suggest that chemical composition of residues of Bt- and non-Bt crops has no significant difference with each other (Folmer et al. 2002; Munga et al. 2005; Lu et al. 2010; Chun et al. 2012; Zhaolei et al. 2017). However, variations observed by some of the researchers might be due to the use of different analytical techniques, age of plant materials used, and events of Bt transformation (Icoz and Stotzky 2008). It is also important that whether rice residues are added to field directly or after composting to enhance the soil fertility. These general agronomic practices significantly increase the effects on soil microorganisms linked with Bt rice cultivation. Temporal shifts in community structure of actively decomposing microbes go together with properties of the transgenic straw during the process of decomposition (Marschner et al. 2011).

7 The Future Framework of Transgenic Plants Impact Assessment on Soil Ecosystem

Experiments conducted to investigate the effects of GM plants on soil microbial community structure and function conclude that observations of different studies contradict each other and a clear gap in the primary information and baseline data regarding the interaction of GM plant and nonliving and living components of soil still exist. This all creates a confusion, which is basically due to absence of universal schematic framework. This approach-based framework is to assess benefits and ecological risks of transgenic crops on soil. This framework must be holistic approach-based which will reflect directly the response of ecosystems of soil to the transgenic plants.

Basically, impact of monitoring concepts of transgenic plants is needed to integrate baseline data of environmental monitoring networks, data of impact assessment, and impact threshold values with impact assessment on transgenic crops in the future. This assessment for future impact must be a long-term assessment and should also be carried out to check herbicide tolerance in transgenic crops. This long-term assessment represents slow change in variables, for example, accumulation and biodiversity of transgenic plants produced in soil. Changes in soil variables specifically ecological (chemical, physical, and biological) which occurs due to these transgenic crops must be prioritized in an integrated framework.

Not only authentic monitoring data can be created by integrated prioritization regarding assessment approach of impact, but this methodology is also helpful in making a fast decision at advanced stages.

The assessment framework of future impact should have the following components:

1. Monitoring of half-life or retention time of products produced by transgenic crops in soil; there will be no effect on soil diversity if these transgenic products have very less retention time. Therefore, positive or negative effects must be monitored in agro-climatic zones of soil ecosystem if these products have high retention time.
2. Monitoring of various changes in temporally and spatially distributed ecological variables of soil.
3. Monitoring network of transgenic and integrated agro-environmental plants.
4. Within monitoring network of transgenic plants, an observation of stimulation or inhibition of micro-organisms activity as a result of differences in composition and amount of transgenic plants-released root exudates in agro-environments.
5. Impact of agriculture practices of agro-ecosystems of transgenic plants on soil processes and diversity.
6. At transgenic crops planted sites, monitoring of transfer of horizontal gene between soil diversity and transgenic crop.
7. Changes in microorganism functions as a result of rare events such as horizontal gene transfer.

Soil health evaluation is based on soil performance and capacity to improve productivity and plant growth. This role of growth promotion must be preserved for use in future. Soil health indicators include several parameters (nutrient retention, fertility, soil organic matter, erosion, etc.), physical (bulk density, soil structure, and infiltration), chemical (soil nitrate, reactive carbon and pH), and biological (microorganisms, their activities and soil enzymes). To find out effect of genetically modified crops on soil, their effect on some abovementioned indicators with insect resistance and herbicide resistance traits has been inquired. But still there is a lack of integrated studies regarding transgenic crops impact on soil quality parameters. Multiple soil parameters must be monitored for impact assessment of transgenic crops on soil health and quality of agroecosystems in the future to make an exact and real picture. Transgenic crops impact will be represented by even a single alteration in function and structure of soil biological representative which may be single species or community in transgenic agroecosystem. Still, no any variety of transgenic rice or Bt rice have been found out for commercialization because of controversy regarding biosafety. However, multiple reports exhibiting impact of Bt rice on microbial composition and activities of soil enzymes in rhizosphere are available (Liu et al. 2008; 2019; Li et al. 2019), but comprehensive study regarding Bt rice risk assessment is lacking.

Microbial species regulate fertility and important functions of soils. Future studies should focus on monitoring function and structure of microbes and their

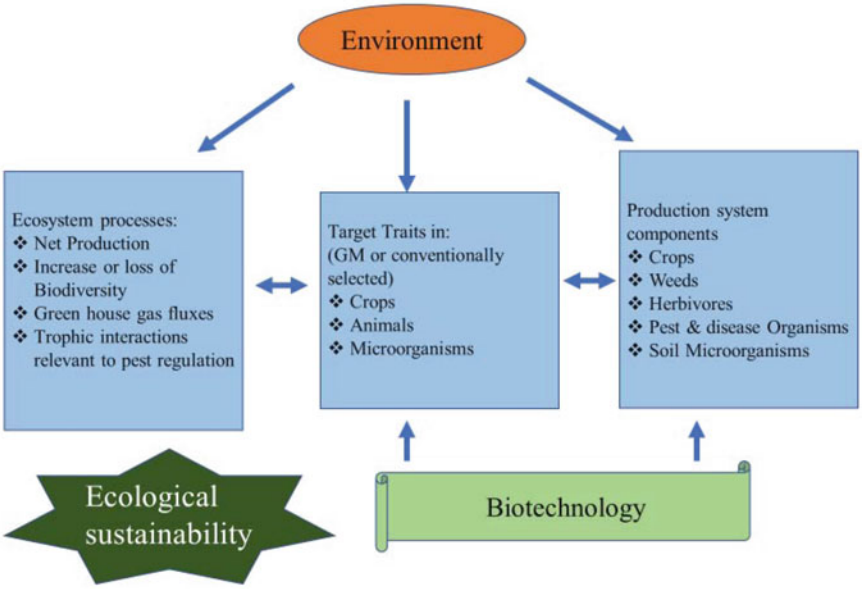

Fig. 3 Influence of genetically modified organisms on agroecosystem processes

communities which will help in finding any negative or positive effect of transgenic plants on soil (Kowalchuk et al. 2003; Bruinsma et al. 2002). The effect of transgenic crops on soil ecosystem can also be recorded through monitoring of biological components, for example, keystone species together with abiotic factors as these show variabilities in functioning in different soils. Moreover, study must be performed to find out impacts on key players in a specific ecosystem. These keystone indicators' selection should be based on ecological significance, perturbations-responsiveness, availability of methods for practical assay, and agronomic relevance. Along with these keystone indicators, broader analysis to find out impact on soil faunal and microbial communities to improve both detection and sensitivity of some unforeseen effects must be performed (Lilley et al. 2006). This monitoring requires various measures such as monitoring diversity, activity, and biomass of faunal and microbial communities (Fig. 3).

Transgenic crops impact on soil can also be indicated by monitoring size of soil microorganism community including collembola and nematode, composition of bacterial communities of rhizosphere, microbial community-metabolic fingerprints, and substrate utilization method. This will help us in making a decision regarding the use of transgenic plants as a sustainable practice in agriculture.

Agriculture practices including irrigation and tillage have an important role in inhibiting and enhancing positive and adverse effects of crops on soil microbes. Various management practices in agriculture associated with especially transgenic crops must be monitored in the future to find out any effect on soil microbial activity. Agriculture practices control contact of transgenic plants biomass and soil

microorganism communities. A significant aspect is horizontal gene transfer in between soil biodiversity and transgenic crop in an agroecosystem that must be monitored regularly through some modern techniques.

A framework usually based on points discussed above will make sure consistent results by redefining evaluation process of transgenic crops impact. This will be helpful for researchers in finding transgenic plants' impact on function and structure of soil microorganisms. Only a small section of microorganisms is culturable and identifiable by using standard methods of analysis. Well-balanced and accurate view of soil system will be obtained by using combining techniques in all cases.

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Genetic Engineering for Developing Herbicide Resistance in Rice Crops

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Abstract

Herbicides are used in modern agriculture to control weeds that pose threat to agricultural crops by competing with them for light and nutrients. Continuous use of the same type of herbicide results in the evolution of weeds that are resistant to herbicides. Indiscriminate use of herbicides can also cause environmental pollution. Development of transgenic rice that is resistant to more than one herbicide is a solution to these problems. The use of herbicide-resistant variety of rice also reduces the cost for weed control. Genetic engineering involves the manipulation of an organism's genetic material using various molecular biological techniques.

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Source of transgenes for herbicide resistance includes bacteria and plants. Development of transgenic rice that is herbicide-resistant will result in the development of wild rice. Genetically engineered rice that is herbicide-resistant has changed the usage pattern of herbicide worldwide. Examples for herbicide-resistant rice varieties include Clearfield, Roundup Ready, and LibertyLink. Clearfield is imidazolinone-resistant, Roundup Ready glyphosate-resistant, and LibertyLink glufosinate-resistant. Despite having so many benefits, genetically engineered herbicide-resistant rice varieties have some drawbacks. One such drawback is that genetically improved crops are also responsible for the problems associated with herbicide-resistant weeds. Another issue is related to the acceptance of genetically modified herbicide-resistant rice variety by the public. It is also not that easy to find domestic and international markets with herbicide-resistant rice varieties. Hence, proper production and marketing strategies should be adopted along with the development of herbicide-resistant rice varieties that are developed using genetic engineering.

Keywords

Oryza sativa · Herbicide resistance · Gene constructs · Marker genes · Clearfield · Roundup Ready · LibertyLink

1 Introduction

Rice, being a staple food crop, is grown in widely divergent, irrigated, and rain-fed uplands and rain-fed lowlands across Asia, Africa, and America (Khush 2004; Kubo and Purevdorj 2004; FAO 2014). Several abiotic and biotic stresses like weed infestation severely affect rice production, which in turn contributes to food insecurity. To a maximum extent, weeds affect the increase in yield of rice (Chauhan 2013).

Herbicides are chemical substance or cultured biological organism that has the ability to either control or suppress plant growth (Table 1). **Herbicides prevent or eliminate weeds and thus reduce manual and mechanical weeding. As weeds compete with crops for sunlight, water, and soil nutrients, herbicides can also prevent soil erosion and water loss.** The development of multiple herbicide-tolerant crops is still in its infancy, and only a few crops with herbicide tolerance traits have been reported and commercialized in recent times. The extensive use of synthetic herbicides to control weeds has imposed strong selection for any trait that enables plant populations to survive and reproduce in the presence of the herbicide that limits food security globally.

Plant growth-promoting rhizobacteria are beneficial bacteria capable of inducing growth and increasing plant tolerance to biotic and abiotic stresses (Mishra and Nautiyal 2012). The inhibition in growth may also be due to a decrease in germination, which can be attributed to a change in enzymatic activity that affects the mobilization of storage compounds during germination, in turn, causing swelling

Table 1 List of herbicides used against certain diseases in rice plants[a]

Name of herbicide	Name of the disease	Causal agent	Symptoms
Thiobencarb	Rice blast	*Pyricularia oryzae*	Leaf blade are elliptical or spindle-shaped and brown lesions on branches
Butachlor	Sheath blight	*Rhizoctonia solani*	Initial lesions are small, ellipsoid, or ovoid and greenish-gray
Alachlor	Brown spot	*Bipolaris oryzae*	Dark brown to black oval spots on grains
Trifluralin	Sheath rot	*Sarocladium oryzae*	Panicles, florets turn red-brown to dark brown
Bensulfuron methyl	Bakanae	*Fusarium fujikuroi*	Plants bear few tillers and leaves dry up quickly
Ethoxysulfuron	Bacterial blight	*Xanthomonas Oryzae*	Yellowish border between dead and green areas of the leaf

[a]Blackie and Conroy (1994), Schil et al. (1994), Hillocks et al. (1995), Sharma and Nagarajan (1997)

response and increasing the peroxidase level in rice plants. Improving soil fertility is the key factor to enhance plant growth and rice yield. The excessive use of chemical fertilizers in the current decades has led to soil toxicity caused by toxic heavy metals, adversely affecting the health of rice plants (Habibah et al. 2011). Microbes have been reported to be a key factor in maintaining soil quality and increasing rice yield and growth. The use of microbes to enhance rice growth while making the plant resistant to pathogens has been reported as an eco-friendly way to maintain the ecosystem. For decades, the application of microbes in a sustainable agro-ecological manner has rapidly increased due to their ability to act as plant growth promoters (Anhar et al. 2011).

2 Origin of Herbicide-Resistant Rice

Weed management has been a vital element of crop farming since the beginning of agriculture. We can see the large loss of financial yield and the quality of products due to weed competition. On the view of increasing population, there is a huge demand of agricultural products (Schulz et al. 2013). To fulfil this agricultural product demand without a major increase of land for farming, it is essential to improve the eminence and productivity of agriculture. Weed management plays an important role for both crop productivity and quality improvement. There are different chemical, cultural, biological, and mechanical methods of weed management applied all over the globe. These methods have both advantages and disadvantages (Burgos et al. 2006). For the sustainable development of agriculture, the use of herbicides for weed control is playing an important role. On the other hand, herbicide's nature will constantly improve that will lead to the creation of further accurate and ever better scientific and environmental criteria for business use. Recently, there are many herbicides that all have different crop safety, toxicology,

spectrum, unit activity, and environmental effect. The wider use of broad-spectrum herbicides such as glufosinate, glyphosate, imidazolinone, and sulfonylurea has also shown to not able to selectively kill weeds (Kishore et al. 1992). In recent years, development and commercialization of plant gene transfer technology created enormous opportunities to introduce herbicide tolerance genes to several rice plants to develop herbicide resistance (Kishore and Shah 1988; Klee and Rogers 1989; Vasil et al. 1990; Pantone and Baker 1991; Croughan 2003; Burgos et al. 2006; Delouche et al. 2007; Schulz et al. 2013).

3 Gene Constructs and Marker Genes for Herbicide-Resistant Rice

Innovations in genetically improved herbicide resistance tools, which commenced with the presentation of former glyphosate-resistant variety of soybean in 1996, launched a new approach to deal with weed growths in agricultural land. Since then, various significant transgenic crops that are resistant to many herbicides have already been grown and commercialized (Green and Owen 2011; Reddy and Nandula 2012). Most of the genetically modified crops have transformed with single herbicide-tolerant markers against basta, glyphosate, PPO, AHAS, or HPPD-inhibiting herbicides. With growing global need for food materials and other agronomic products, herbicide resistance technology has subsidized intensely during the previous few decades, though the continuous overdoing of a particular herbicide several times in a breeding season enhances the potential threat of maximum progression of resistant weeds which has become a foremost concern for agriculture industry worldwide. Additional, higher prescribed amount of herbicide treatments cause high risks to crop fields and environment (Owen and Zelaya 2005; Bhat and Chopra 2006). Most of the weed varieties that are tolerant to several herbicides have been recognized all over the world (Tranel and Wright 2002; Owen and Zelaya 2005; Green 2009). It is very crucial to retain variety in herbicide usage for competently exploiting the properties of currently existing vital herbicides to cope the weeds adeptly for long term. The advancement of genetically modified crops that are tolerant to two or more herbicides endures a main challenge and significant space for agricultural programs.

Most widely used marker *bar* gene construct was exposed in the early 1970s from *S. hygroscopicus* strain Tu 494 and *S. viridochromogenes*. *S. hygroscopicus bar* gene of phosphinothricin acetyltransferase (PAT) with 85% DNA sequence homology with *pat* marker gene from *S. viridochromogenes* confers resistance to the herbicide phosphinothricin (Bayer et al. 1972; Kondo 1973). The bacterial *bar* gene detoxifies phosphinothricin by acetylating its amino group, thereby helping in the survival of the plants (Reddy et al. 2011). The *bar* transgenic lines also exhibited resistance to bialaphos up to 500 ppm (Christou et al. 1991) indicating its potential against wide range of herbicides. The *bar gene* has been significantly implemented in growing glufosinate-tolerant rice (Oard et al. 1996; Xiao et al. 2007; Xiao 2009; Tian et al. 2015). The *bar* transgenics belonging to diverse rice varieties

demonstrated different intensities of glufosinate tolerance (Oard et al. 1996). It is predicted that the reason for differential response of PAT proteins extracted from diverse bacterial strains that may have altered kinetic constants leading to distinctive glufosinate tolerances in different plant cells or cellular compartments (Yun et al. 2009). To overcome the issue of differential response of PAT gene construct isolated from diverse strains, a novel recombinant PAT gene was developed by codon optimization showing high tolerance to glufosinate as well as providing a glufosinate-tolerant rice collections with potential agronomic applications (Cui et al. 2016b).

Protoporphyrinogen oxidase (protox) catalyzes oxidation of protoporphyrinogen IX to protoporphyrin IX in transitional step of biosynthetic pathways of hemes and Chi (Beale and Weinstein 1989). The protox is inhibited by peroxidizing and diphenyl ether herbicides resulting in excessive accumulation of Proto IX. Proto IX being a photosensitizer produces reactive oxygen species stimulating membrane lipid peroxidation resulting in cellular death through light-dependent procedure (Duke et al. 1991). The overexpression of protox transgene results in elevated accumulation of protox chloroplast and tolerates cellular damage by lipid peroxidation and electrolyte leakage (Ha et al. 2004). Protox genes from bacterial as well as plant sources have been used for development of herbicide-resistant rice cultivars. Protox genes from *Bacillus subtilis* and *Myxococcus xanthus* transformed into rice plants have shown better tolerance to the herbicide oxyfluorfen (Ha et al. 2004; Jung et al. 2004; Jung and Back 2005).

A mouse dihydrofolate reductase (*dhfr*) gene modified for expression in plants conferring methotrexate resistance was introduced into rice protoplasts (Meijer et al. 1991) in suspension culture. The transgenics showed very high level of resistance to methotrexate. The *dhfr* is an underutilized gene for development of herbicide resistance in spite of its immense potential to tolerate herbicide stress at high concentrations (Table 2, Fig. 1).

4 Agronomic Performance of Herbicide-Resistant Rice

Nowadays, in genetically modified herbicide-resistant crops (HRCs), the main focus is on food safety and genetic contamination issues (Kuiper et al. 2001; Messeguer 2003; Bawa and Anilakumar 2013) and whether the agronomic performance of such crops is affected greatly (Carpenter 2010). Herbicide resistance providing gene traits apparently reflect an advantage in the presence of the herbicide. Nevertheless, lack of herbicide may deliberate resistance cost in genetically modified (GM) crops in comparison to sensitive wild-type analogs (Purrington and Bergelson 1999; Vila-Aiub et al. 2009).

There are several factors which may account for the entire yield difference between GM and non-GM crops, particularly the resistance gene, mutation, number of mutant alleles, genetic background, and environmental conditions (Roux et al. 2004; Yu and Powles 2014; Vila-Aiub et al. 2015). Several biochemical changes at the herbicide target enzyme hampering enzyme catalytic capacity, reducing substrate

Table 2 List of gene constructs and marker genes reported and used for developing herbicide resistance in rice plants (Adapted and modified from Kathuria et al. 2007)

Cultivar/variety	Gene constructs and marker genes	Method of transformation and level of resistance	References
Gulfmont, IR54, IR26, IR36, IR72	p35S-bar-RBCSt	Bombardment for resistance to bialaphos up to 500 ppm	Christou et al. (1991)
Taipei 309	p35S-DHFR-nost	Polyethylene glycol-mediated transformation for high level of resistance to methotrexate	Meijer et al. (1991)
IR72	p35S-bar-35St	Polyethylene glycol-mediated transformation for resistance to Basta	Datta et al. (1992)
Gulfmont, IR72	p35S-i (ADH1)-bar-nost	Bombardment for resistance to glufosinate at field level	Oard et al. (1996)
Nackdong	pUBI1-protox-nost, pUBI1-TS-protox-nost	*Agrobacterium*-mediated transformation for resistance to oxyfluorfen	Lee et al. (2000)
Red rice Nortai	*bar* gene	Tolerance to glufosinate (liberty™)	Oard et al. (2000)
Nipponbare	p35S-CYP2C9/C19-nost	*Agrobacterium*-mediated transformation for transgenic plants evade sensitivity to herbicides	Inui et al. (2001)
Nipponbare	p35S-CYP2B6/C9/C18/C19-nost, pE7-AMV5 UTR-CYP1A1-nost	Polyethylene glycol and *Agrobacterium*-mediated transformation for cross tolerance to several herbicides	Ohkawa and Ohkawa (2002)
Nipponbare	p35S-CYP1A1-t, p35S-CYP2C19-t, p35S-CYP2B6-t	*Agrobacterium*-mediated transformation for strong cross-tolerance to various herbicides	Kawahigashi et al. (2002)
Notohikari	pE35S-cbnA-35St	*Agrobacterium*-mediated transformation for enhanced degradation of chlorinated compounds	Shimizu et al. (2002)
Lemont	p35S-AsOSGSTIII-GSTt	*Agrobacterium*-mediated transformation for reduced detoxification of pretilachlor and phenolics	Deng et al. (2003)
Dongjin	pUBI-mx-protox-nost	*Agrobacterium*-mediated transformation for tolerance to oyfluoren	Jung et al. (2004)
Nipponbare	p35S-CYP2B6-nost	*Agrobacterium*-mediated transformation for enhanced detoxification of several kinds of herbicides	Hirose et al. (2005)
Nipponbare	p35S-CYP1A1-nost, p35S-CYP2B6-nost, p35S-CYP2C19-nost	*Agrobacterium*-mediated transformation for cross-tolerance to many herbicides	Kawahigashi et al. (2005a)

(continued)

Table 2 (continued)

Cultivar/variety	Gene constructs and marker genes	Method of transformation and level of resistance	References
Nipponbare	p35SE7-AMV5'UTR-CYP2b22, CYP2C49-nost	*Agrobacterium*-mediated transformation for broad-spectrum tolerance toward herbicides	Kawahigashi et al. (2005b)
Nipponbare	p35S-AMV5'UTR-CYP2B6, CYP1A1, CYP2C19-nost	*Agrobacterium*-mediated transformation for enhanced tolerance	Kawahigashi et al. (2006)
A *japonica* rice variety, Taichung 65	pGEX2T-ALS-G95A mutated ALS gene	*Agrobacterium*-mediated transformation for tolerance to pyrimidinyl carboxy herbicides	Okuzaki et al. (2007)
Nipponbare	W548L/S627I	*Agrobacterium*-mediated transformation for resistance to PC herbicides	Kawai et al. (2007a)
Kinmaze	OsALS-W548L/S627I	Polyethylene glycol-mediated transformation for high level of resistance to the PC-type ALS-inhibiting BS	Kawai et al. (2007b)
Mutated rice ALSs	W548L-and S627I-mutated ALS gene	Polyethylene glycol-mediated transformation for tolerance to distinct classes of herbicides including sulfonylureas, imidazolinones, pyrimidinylcarboxylates, triazolopyrimidine sulfonamides, and sulfonylaminocarbonyltriazolinones	Kawai et al. (2008)
Indica-derived cultivars, *indica* cultivars, and *japonica* cultivar	OsmALS W548L/S627I	*Agrobacterium*-mediated transformation for tolerance to BS	Taniguchi and Kawata (2010)
Rice cultivar Xiushui-110 (*Oryza sativa* L. ssp. *japonica*)	pG6-Ubi	*Agrobacterium*-mediated transformation for tolerance to glyphosate	Zhao et al. (2011)
Rice calli derived from seed of own cultivars	OsmALS (W548L/S627I	*Agrobacterium*-mediated transformation for tolerance to BS	Endo et al. (2012)
A *japonica* PGMS rice	pM19-Epsps, pC3300-ubi-Ω-OsbHLH1 (with *bar* gene)	*Agrobacterium*-mediated transformation for dual tolerance to glyphosate and glufosinate	Deng et al. (2014)
IR64	CaMV35S:Os-mEPSPS:PolyA	*Agrobacterium*-mediated transformation for tolerance to glyphosate	Chandrasekhar et al. (2014)

(continued)

Table 2 (continued)

Cultivar/variety	Gene constructs and marker genes	Method of transformation and level of resistance	References
Indica rice cultivar IR64	pCAMBIA-ubimCP4-EPSPS	*Agrobacterium*-mediated transformation for tolerance to glyphosate	Chhapekar et al. (2015)
Japonica rice cultivar Kitaake	p35S:OsALS	Bombardment for tolerance to BS	Li et al. (2016)
Japonica cv. Nipponbare	pCXUN-Cas9-gRNA1 pCXUN-Cas9-gRNA2	Bombardment for tolerance to chlorsulfuron and BS	Sun et al. (2016)
Japonica and *indica* rice cultivar	pU130-$aroA_{J.sp}$	Electroporation for tolerance to glyphosate	Yi et al. (2016)
HTM-N22 (Nagina22)	AHAS (Acetohydroxy acid synthase)	Ethyl methane sulphonate-induced mutagenesis approach for tolerance to imazethapyr	Shoba et al. (2017)
Indica rice cultivar Swarna	Mutant OsmAHAS gene along with the *bar* gene	*Agrobacterium*-mediated transformation for dual tolerant to bensulfuron methyl and glufosinate	Fartyal et al. (2018)
Japonica rice variety (JD164)	Mutated AHAS	Single mutation (S627N) in AHAS for tolerance to imidazolinone	Piao et al. (2018)

PGMS photoperiod-sensitive genic male sterile, *AHAS* acetohydroxy acid synthase, *ALS* acetolactate synthase, *BS* bispyribac-sodium

affinity, and/or altering feedback inhibition which accounting for a resistance cost that may be activated by herbicide resistance point mutations (Yu et al. 2010; Han et al. 2012).

Recent reports on some herbicide-resistant rice cultivars have shown significant modifications in growth and yield aspects (Qaim and Zilberman 2003; Klümper and Qaim 2014; Schütte et al. 2017). Whether alteration in the rice genome leads to a relatively higher agronomic performance is still ongoing, and there is debate among researchers.

In developing countries, GM technology not only enhances crop yields but also renders other environmental benefits such as conservation of natural habitats as less land may be required for agriculture (Wesseler et al. 2011). Areal et al. (2013) analyzed yields of GM crops for developing and developed countries and concluded that GM crops serve better than their regular counterparts in agronomic perspectives in developing countries than developed countries. The main conclusion they present is that adoption of GM crops is both economically and agronomically advantageous over their conventional counterparts. Some other studies reported that yield between GM and conventional crops does not differ (Qaim 2009).

As mentioned above, why herbicide-resistant GM crops are expanding worldwide may be partially explained by the greater agronomic performance associated

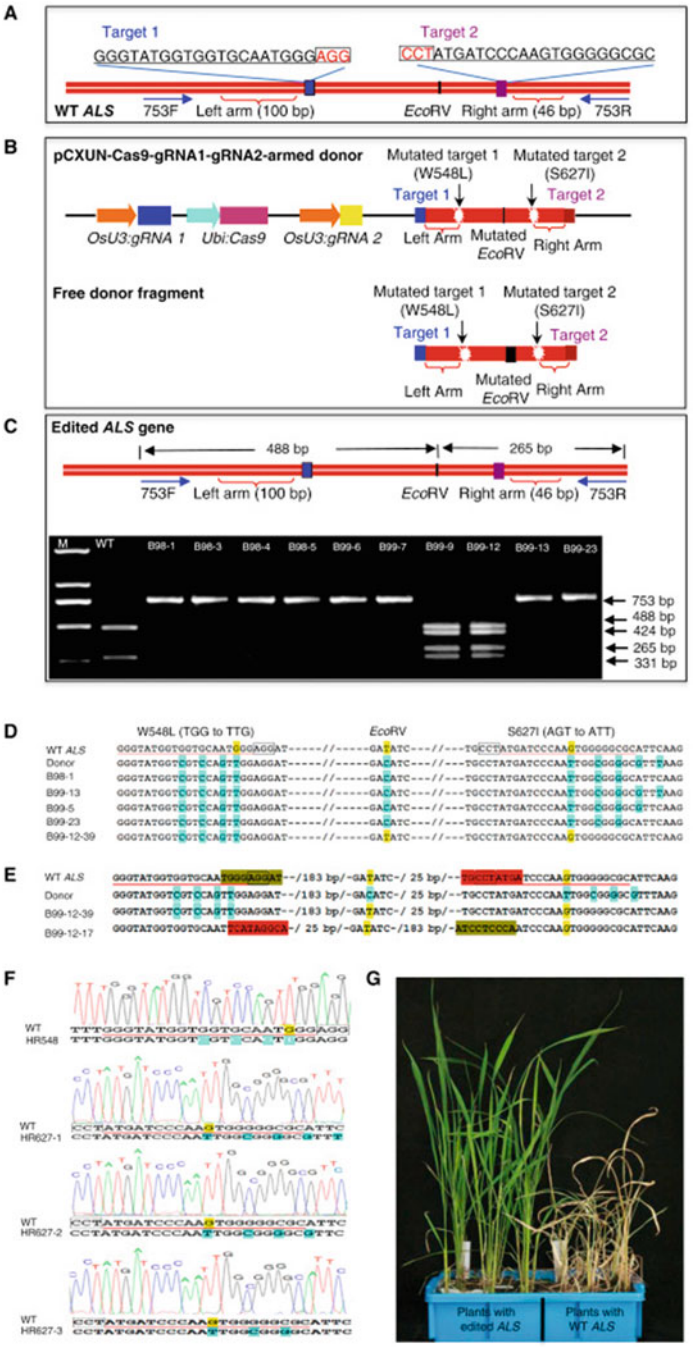

Fig. 1 Use of CRISPR/Cas9-mediated homologous recombination for the production of herbicide-resistant rice. (**a**) Wild-type ALS gene; (**b**) construct to produce Cas9 protein; (**c**) PCR amplification by primers 753F/R; (**d**) sequences of HDR types; (**e**) sequences of two clones from B99–12; (**f**) HDR chromatogram of modified ALS gene in desired region; and (**g**) 36 days after sprayed with

with them. However, the debate on whether adopting GM crops leads to a relatively higher agronomic performance is yet continuing. Compared with their conventional rice counterparts, GM crop may be lower, similar, or greater in fitness. Therefore, fitness of genetically modified herbicide-resistant rice in comparison with their conventional counterparts/wild type would be discussed based on the evidence from the literature.

4.1 Clearfield Rice

Since the introduction of Clearfield rice in 2002 (Croughan 2003), there are several research on its agronomic performance in comparison with conventional cultivars. It has been reported that Clearfield rice grain yields were lower than conventional cultivars (Sha et al. 2007; Shivrain et al. 2009; Deliberto and Salassi 2010; Sudianto et al. 2013; Shengnan et al. 2016). Despite this, Clearfield cultivars showed greater head rice yield than their regular counterparts. In addition, no notable difference in ratoon yield was noticed between Clearfield rice and conventional rice. When compared with conventional rice, Clearfield rice possessed the same maturity and plant height but greater seedling vigor. Lyman and Nalley (2013) found significant yield advantages for all three hybrid cultivars tested over the best-performing conventional alternative.

4.2 Roundup Ready Rice

It has been reported that the insertion of foreign genes affects the fertility of plants (Yasuor et al. 2006). However, transgenic hybrid rice varieties had greater fitness when compared to its conventional counterpart due to the lack of glyphosate application (Wang et al. 2014). They concluded that these fitness advantages in transgene-bearing individuals would lead to higher competitiveness against weedy rice compared with nongenetically engineered GE rice. Likewise, other researchers have reported faster germination and taller plants in transgenic glyphosate-resistant rice against conventional ones (Goulart et al. 2012; Shivrain et al. 2006).

On the other hand, Han et al. (2017) reported reduced agronomic performance in glyphosate-resistant rice due to altered concentration of carbon metabolites as a consequence of disturbance of shikimate and then other related metabolic pathways. A study revealed that the fertility and main agronomic characteristics of the transgenic line remain unaffected upon the simultaneous transformation of the herbicide-resistant genes *bar* and *Epsps* into a transgenic line 7001S. The agronomic evaluation of glyphosate tolerance in transgenic rice lines revealed that glyphosate is rarely affected based on overall confirmation (Cui et al. 2016a).

Fig. 1 (continued) 100 μM BM: left side with edited ALS gene and right side with wild type [Reproduced with permission from Sun et al. (2016), Copyrights reserved to Elsevier Inc., 2016]

4.3 LibertyLink Rice

In LibertyLink rice, the overexpression of a protox gene reported likely to redirect energy and fix carbon resources apart from grain production. However, some controversial results have been reported (Hareau et al. 2005; Sudianto et al. 2013). While comparing with its non-transgenic counterparts, transgene in LibertyLink rice was found to be associated with lesser plant height and maturity with significant change in fitness outcomes (increased, decreased, or none) based on trait (Oard et al. 2000; Lu et al. 2016).

Schuh et al. (1993) reported that the introduction of the nptII gene in rice followed reduced fertility, late maturity, and smaller flag leaves to their non-transformed, protoplast-derived counterparts. According to Cui et al. (2016b), in the absence of glufosinate application, the transgenic rice plants were found to exhibit similar panicle length and charged grain rate when compared to their corresponding non-transgenic plants. Furthermore, the agronomic performances of transgenic and their regular counterparts exhibited no statistical difference. But there were notable differences in 1000 grain weight, number of panicles per plant, and plant height between homozygous and non-transgenic plants. They concluded that homozygous transgenic plants also showed changes in various aspects of agronomic characteristics, except to provide glufosinate resistance to the transgenic rice. This was further established by the agronomic performances of transgenic plants under glufosinate applied at tillering stage.

By comparison of growth and yield of wild-type and transgenic rice in paddy fields, Jung et al. (2010) showed a minor yield drag in nontreated transgenic plants relative to the nontreated wild-type plants. Moreover, the protox-resistant transgenic rice, wild type, and conventional plants showed similar heights and number of tillers with an acceptable yield difference, although the number of panicles per plant of transgenic rice was found to be greater as compared to wild type and regular plants.

Oard et al. (1996) after performing trials under non-weedy conditions recorded grain yield range between highest and lowest grain yielding transgenic lines as 1.12 and 2.24 kg/ha rates. Their investigations showed some untreated transgenic lines produced grains lesser than the untransformed parental cultivars, but approximately 80% of the lines produced is equal or better than the control plants. Further, heading rates were almost equal or only slightly higher in the absence of herbicide treatment when compared to the non-transgenic controls. Oard et al. (1996) analyzed variability for all features seen within the transgenic lines in the absence of herbicide to determine the impacts of integrated transgenes.

As reported by Jung et al. (2010), the protox-resistant transgenic rice and wild-type plants possessed similar heights, and a number of tillers and variations in their yield production were usually negligible (7–8% reduction in yield of transgenic to that of wild type). They correlated this reduction with the reduced spikelets per panicle and 1000 seed weight. However, the number of panicles per plant of transgenic rice was higher than those of wild type. Finally, neither the advantages nor the disadvantages of genetically modified herbicide-resistant crops are certain or universal and are dependent on a wide range of factors, particularly region, on weed

infestation levels, as well as on seed and technology costs. GM profits are usually higher than those achieved by conventional varieties due to the combination of yield protection and lower production costs. Indeed, herbicide transgenic technologies do not increase yields significantly, but their use facilitates more economic weed control measurements. As a rule, under high weed infestation levels as is the case, paddy field farmers would benefit from GM herbicide-resistant rice adoption.

In conclusion, GM rice as a whole give higher yields than non-GM counterparts across the globe although there are some doubts about the performance of GM crops relative to conventional ones. Therefore, further investigation is needed on the mechanisms responsible for bringing the slight yield variation between various herbicide-resistant transgenic rice and their non-transgenic controls.

5 Impact of Herbicide-Resistant (HR) Rice Technology

Transgenic biotechnology that uses co-transformation with multiple transgenes is measured to be the most capable tool of the twenty-first century (Halpin 2005). Several transgenic plants with a number of important herbicide resistance transgenes have been successfully developed through genetic engineering (Lutz et al. 2001; Toyama et al. 2003). In rice, there are three HR systems that have been produced, namely, glyphosate-, glufosinate-, and imidazolinone-resistant varieties (Gealy et al. 2003). Glyphosate- and glufosinate-resistant rice, produced through transgenic tools, are capable of providing resistance to these nonselective, broad-spectrum herbicides, whereas imidazolinone-resistant rice is produced by chemical mutagenesis and provides resistance only to the imidazolinone herbicides (Gealy et al. 2003). There are several possible positive and negative impacts of using HR rice technology that are discussed below.

5.1 Impact on Food Security and Biosafety Issues

Rice yields, produced through conventional breeding approach, despite great advancement, do not appear as sufficient to meet the demands of growing populations of the world, mainly in developing countries (Conway 1997; FAO 2004). Novel transgenic technologies have great potential to widening the available genepool that offers enormous possibilities for rice breeding and food security at global level (FAO 2004; Brookes and Barfoot 2013). However, weeds affect rice yields by reducing nutritional uptake and space among other factors. HR technologies have increased rice yields by broadening the prospects of herbicide use (Marra and Piggott 2006), decreasing crop phytotoxicity, and improving the control of problem weeds such as wild and weedy rice (Olofsdotter et al. 2000; Lanclos et al. 2003). Oard et al. (1996) reported an increase (up to 7%) in the rice yield due to more weed control by HR rice.

The commercial production of HR rice may raise food security by enhancing rice yields. Liu et al. (2008) reported that the commercialization and wide environmental

release of transgenic crops have developed many biosafety measures like food and feed safety, environmental safety, and accessibility of biodiversity. Nowadays, biosafety issues become a crucial part of the development and application of GM crops, and potential environmental impacts are the most debated among them (Pretty 2001; Ellstrand 2003; Lu and Snow 2005; Wang et al. 2006). Hence, it becomes mandatory to face the problems of these biosafety issues by using a solid science-based study.

5.2 Impact on Farmers and Consumers

In general, herbicide-resistant rice provides farmers an easy and flexible approach for managing weed problems (Duke 2005). However, it was reported in a glyphosate-resistant crop the costs in weed management plans by both the conventional and HR crops reduced due to decrease in the costs of herbicides. Hence, the adoption of genetically modified HR rice by farmers may offer superior economic gain in comparison to that by conventional crop and herbicide programs. For example, the adoption of glyphosate-resistant crops is reported to save US farmers about $1.2 billion allied with the prices of conventional herbicide management including purchases, application, tillage, and hand weeding.

The estimated cultivated area of GM crops including rice has extended up to 130 million hectares and produced approximately US$52 billion economic benefit globally by the end of the year 2009 (James 2009). However, with the continuous growing human population that will reach 9.2 billion by 2050 and the global requirement for rice will also be increased crossing the current production capacity, which further requires new technologies to fulfill the demand of rice yield (James 2009). On the other hand, the selection of GM crops may cause some constraints to farmers due to the nonavailability or increased prices of seeds as well as the technology involved and the absence of suppliers (EU 2000).

5.3 Environmental Impact

HR rice plants may be useful to the environment because of no-tillage system that reduces soil erosion or gives further weed management enhancing biodiversity in the area. However, it is necessary to emphasize that the risk from these plants should be thoroughly assessed before releasing them particularly if they are of weedy nature or may outcross to similar weeds. The environmental impacts of many HR crops can be evaluated from previously available data on the crop biology, the presence of suitable wild or weedy relatives, and the transgene phenotype (Hancock 2003).

5.4 Weed Control and Management

Weeds are very critical production constraints in any agricultural system including rice and other crops as they rigorously compete with crops nutrient and other vital supplies (Tawaha and Turk 2001; Turk and Tawaha 2001, 2002a, b, 2003; Tawaha et al. 2002; Tawaha et al. 2003; Al-Tawaha and Seguin 2006). Their introduction forces a critical challenge to crop yield, richness, and survival. Dependence on herbicides for weed control is supposed to continue due to the absence of any admissible superior technology (Duke et al. 1993). Herbicides act as the crucial constituents of the current integrated weed control program. Adoption of HR rice may conquer the obstacles related to weed management and promote the adoption of resource conserving techniques (Malik et al. 2003). The introduction of HR rice is usually put forward to improve control of the weedy vegetation associated with this crop, particularly of red rice and other weedy rice (Olofsdotter et al. 2000; Gealy and Dilday 1997), as well as to implement an alternative mean for the control of weeds that have previously developed resistance to selective herbicides, especially grasses such as *Echinochloa* spp. (Olofsdotter et al. 2000; Wilcut et al. 1996). Herbicide-resistant rice may also allow for the replacement of some of the currently used herbicides by others that are less damaging to the environment (Olofsdotter et al. 2000; Kumar et al. 2008). Since prolonged herbicide use produces resistant weeds, Fartyal et al. (2018) developed dual herbicide-tolerant transgenic rice against bensulfuron-methyl (BM) and glufosinate tolerance that provides more information to crop herbicide tolerance and to sustainable weed control in modern agricultural practices. Moreover, existence of numerous important risks related to HR rice that must be checked before its public adoption should be promoted (Kumar et al. 2008).

5.5 Impact on Soil, Water, and Air

As mentioned above, the HR crops are capable of weed management, which provide zero tillage and thereby minimize soil erosion (Duke 2001; Holland 2004). Studies have been recorded that both glyphosate and glufosinate are highly water-soluble, but they do not leach notably to groundwater, and also glyphosate pollutes surface water lower than other herbicides (Carpenter et al. 2002), and further it evaporates more quickly than most of the other herbicides (Solomon and Thompson 2003; Peterson and Hulting 2004). It has been reported that herbicides can contaminate the air by drift or volatility. Some herbicides such as glyphosate and glufosinate are basically not volatile at room temperature (25 °C) (Lawrence 2002) and have not been found to cause any atmospheric pollution (Van Dijk and Guicherit 1999).

5.6 Impact on Native Biodiversity

Biodiversity in the area may be influenced by the herbicide, to which the herbicide-resistant crop is resistant, is used at a higher level of efficacy for obtaining increased

weed control. The potential reduction in the native biodiversity has been shown by crops with resistant traits either by inducing weediness or by the enhanced destruction of possible wild relatives (Kumar et al. 2008). When the HR crops grow at the center of their genetic origin, then there is a higher risk of decline in the genetic diversity of the same crop, and change in the native species is also expected (FAO 2001). In India (one of the centers of biodiversity of wild rice), the biodiversity risks associated with herbicide-resistant rice are particularly more severe (FAO 2001). The rice populations that are small and rare, facing greater risk, may extinct by swamping. Swamping has been found to be associated with the expected loss of *O. rufipogon* ssp. *formosana*, a wild relative of rice in Taiwan (Kiang et al. 1979; Ellstrand et al. 2000).

5.7 Impact on Herbicide Usage

The HR rice technologies may have the labor-saving advantages over traditional chemical control methods without any accompanying hazards of phytotoxicity. Further, it contributes in the control of some common problematic grass weeds such as weedy and wild rice (Olofsdotter et al. 2000; Lanclos et al. 2003) and also reduces environmental traces of chemical weed control (Nelson and Bullock 2003; Sanvido et al. 2007; Devos et al. 2008). HR rice may present an important and environmentally beneficial option for rice farmers to control weeds effectively and economically. It has been a matter of debate regarding HR crop with enhanced herbicide application. Benbrook (2001) has reported that HRCs have the ability to increase herbicide use. Moreover, Heimlich et al. (2000) while studying on adopted HRCs in the USA have recorded that there is no significant difference in the volume of herbicide used and concluded that this can replace more toxic and persistent nearly double as long as glyphosate (Heimlich et al. 2000).

5.8 Impact on Nontarget Organisms

A number of herbicides are used by spraying which causes their transfer to nontarget sites (Ellis and Griffin 2002; Ellis et al. 2003; Blackburn and Boutin 2003). Also, the reduced or no-tillage system with HRCs has been found to induce more vegetation in a particular area and sometimes makes an improved habitat for other plants and animals. Some reports have been suggested that the herbicides related with HR crops can be toxic to various microorganisms as well as plant pathogens (Toubia-Rhame et al. 1995; Wyss and Muller-Scharer 2001).

5.9 Impact on Volunteers and Crop Rotation

A plant that grows as the subsequent crop or year in a similar field from the seeds lost during the harvest of HR crops is known as volunteer crop (Warwick and Stewart

2005). Gealy (2005) reported that the presence of HR volunteer rice may demand the use of additional herbicides to manage volunteers. Some reports suggested that the problem of rotating crops may be handled with HR technology by making two crops in rotation resistant to the same herbicide (York et al. 2004; Deen et al. 2006).

5.10 Transgene Flow and Weedy Rice Evolution

As reported by Lu et al. (2003), the geographical distribution data informs the gene flow from cultivated rice to wild relatives. Weedy rice may obtain trait of transgenic herbicide resistance easily. Olofsdotter et al. (2000) reported weedy rice that are likely to be an annoying weed in many parts of the world such as the wild species *O. barthii* and *O. longistaminata* or weedy species from cultivated *O. glaberrima* are known to be among the worst weeds in West Africa, whereas rice species such as *O. granulata*, *O. officinalis*, *O. rufipogon*, and *O. nivara* are weedy or wild species in Southeast Asian countries.

6 Conclusion

Farmers could minimize the application of synthetic chemicals and poor weed management by practicing the use of herbicide-resistant rice. However, herbicide-resistant rice varieties could become an issue due to its resistance to broad range of herbicides. Gene flow from herbicide-resistant trait to the wild crop is possible as these are sexually compatible with similar flowering time and are having sympatric range. Prevention of gene flow between herbicide-resistant traits to wild crop could be achieved by applying both conventional and molecular breeding. Before commercializing the herbicide-resistant rice, it is necessary to do thorough risk assessment, particularly in the areas where intercrossing weedy *Oryza* species infestation takes place. In addition, regulators should balance the risks and benefits of the herbicide-resistant rice according to the local people's needs before its release into the commercial market. Thus, herbicide-resistant varieties could be considered as one of the components of integrated weed management.

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An Insight into the Factors Regulating Flowering in Rice: From Genetics to Epigenetics

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Abstract

Yield in rice is determined by the reproductive phase which is determined by the flowering time. Flowering in rice occurs toward the end of the vegetative phase. However, flowering in rice is affected by several factors like environmental atrocities, pathogen attack, etc. The signaling cascade involved in flowering is a complex process that involves coordinated regulation of several genes like Heading date 1 (*Hd1*), Heading date 5 (*Hd5*), and Heading date 6 (*Hd6*) that delays flowering under long day. While genes like *Oryza sativa* Phytochrome B (*OsPhyB*), *Oryza sativa* Constans-like 4 (*OsCOL4*), Supernumerary bract (*SNB*), and *Oryza sativa* Indeterminate Spikelet 1 (*OsIDS1*) inhibit flowering independent of day length. The accumulation of inhibitors of flowering reduces toward the end of vegetative phase, and this is the signal for flowering. Hd1 switches to flowering inducer under short-day conditions with accumulation of LD-specific inducers like *OsMADS50* and *OsDof12*, *OsId1*, *Ehd4* and *miR172*. Alongside, transcription factors, circadian clock genes, photoreceptors, and epigenetic regulation, are also involved. The current review provides an overview of all the factors involved in initiation and regulation of flowering that finally determines the yield potential of rice.

Keywords

Chromatin · Florigens · Histone · Photoperiod · QTL

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1 Introduction

Rice being a staple crop feeds more than half the world population. Due to its sensitivity to various abiotic and biotic stresses, it is imperative that we need to maintain a sustained productivity and high yield potential for securing food for the future. Under the current scenario of global climate change, there has been a substantial reduction in rice yield, and hence it has become very necessary to develop climate resilient rice variety. Yield can be maximized by developing a clear understanding about flowering in rice. The flowering in rice mostly occurs under short-day (SD) conditions but can eventually flower under long day (LD) as well. There are several factors like transcription factors, epigenetic regulation, microRNAs, and circadian rhythm that impact flowering. At the initial stage of development, flowering is inhibited to enable the vegetative parts to grow. Flowering is induced when an adequate amount of transcript for Ehd1 or other florigens have accumulated.

2 Florigens

These are small hormone-like molecules produced in the leaf that are known to control flowering in the bud or in the shoot apical meristem. *Hd3a* is a known florigen in rice identified initially from QTL analysis using a population derived from a cross between Nipponbare (photoperiod-sensitive) and Kasalath a photoperiod-insensitive cultivar (Yano and Sasaki 1997a). *Hd3a* is homologous to *Arabidopsis* Flowering Locus T (FT) as observed when a genomic DNA fragment from Kasalath containing the gene is introduced into Nipponbare and shown to induce early flowering (Kojima et al. 2002; Monna et al. 2002). Of the 13 rice proteins homologous to *FT*, *RFT1*, and *FTL1* are known florigens that when overexpressed in rice are known to induce flowering at callus induction stage (Monna et al. 2002; Izawa et al. 2002; Hori et al. 2013). Similar results were observed where flowering was delayed for 300 days after sowing when RNAi of both Hd3a and RFT1 were overexpressed in rice (Komiya et al. 2009). Contrasting roles have been observed for Hd3a and RFT1 with the latter having a preferential role under SD with RNAi plants of Hd3a showing delayed flowering in comparison to RFT1 RNAi plants that showed flowering similar to non-transformed plants, but under LD conditions, reverse phenotype was observed (Komiya et al. 2009; Komiya et al. 2008; Tsuji et al. 2008). Another interesting observation that came from the study was that RFT1 functions as a florigen when Hd3a is suppressed. Delayed flowering has been observed in Nonabokra. Analysis of the RFT1 from Nonabokra identified an amino acid substitution from E to K at 150th position along with polymorphisms at the promoter region and hence reducing its expression (Ogiso-Tanaka et al. 2013). Florigens when produced in leaves are translocated to SAM where they induce reproductive developments by interacting with 14-3-3 proteins GF14b and GF14c and the complex then interacts with *OsFD1* (Purwestri et al. 2009; K-i et al. 2011; Tsuji et al. 2011). Crystallographic studies have identified that

the complex comprises GF14c (14-3-3 protein) dimer and two molecules of each of Hd3a and OsFD1. The complex together binds to the promoter of *OsMADS15* and that subsequently induces the expression of target genes (Tsuji et al. 2011), though *OsMADs14* and *OsMADS15* have been identified as an inducer of flowering but GF14c acts as an inhibitor.

3 Hd1 and Ehd1: Florigen Regulators

Heading date 1 (*Hd1*) identified from QTL mapping is an ortholog of *Arabidopsis* Constans (*CO*) (Yano and Sasaki 1997b; Lin et al. 2000). It has been observed that plants with nonfunctional *Hd1* or *hd1-1* mutants flowered late under SD but flowered early under LD suggesting Hd1 as an activator/inhibitor under SD/LD conditions. The exact mechanism as to the signaling cascade is unknown, but a hypothesis is that it can bind to different targets under different conditions. However, when Hd1 is overexpressed in rice, it shows delayed flowering under both SD and LD, but this phenotype is not observed in osphyB mutant suggesting that *OsPhyB* has an important role in downregulating the expression of *Hd1* (Ishikawa et al. 2011). Being a member of CO-like protein family, it also contains CCT domain and conserved B-box zinc fingers (Robson et al. 2001; Griffiths et al. 2003a). It has been observed that Early heading date 1 (*Ehd1*) a member of B-type response regulators and involved in cytokinin signaling control the expression of *Hd3a* and *RFT1*. Moreover, the binding efficiency of Ehd1 to its downstream targets is reduced by substitution of Gly to Arg in the binding domain (Doi et al. 2004).

4 Regulators Under LD and SD Conditions

There are several factors inhibiting the flowering during the early developmental stages in rice. Among these factors are genes like Grain number, plant height, and heading date 7 (*Ghd7*) that encode for a repressor that preferentially affects LD. Zhenshan 97, when introgressed with Ghd7 allele from Minghui showed delayed flowering but enhanced yield (Yu et al. 2002; Xue et al. 2008). *Ghd7* is expressed in the phloem and belongs to the CO family and is believed to control the expression of Ehd1 by reducing its expression. In addition, Ghd7 expression is controlled by phosphorylation with a casein kinase-1 protein *Hd16* (Heading date 16, 23). There are several allelic variations for *Ghd7* like *Ghd7-1*, *Ghd7-2*, *Ghd7-3*, *Ghd7-0*, and *Ghd7-0a*, and among them, Ghd7-0 and Ghd7-0a are nonfunctional alleles which are primarily expressed in high latitude cultivars, while the rest are present mainly in low latitude cultivars. These observations thereby clearly suggest an important role for Ghd7 in domestication of rice. Besides the aforementioned, there are several other genes or QTL like Heading date 5 (*Hd5*), grain yield and heading date 8 (*Ghd8*)/Late Heading Date 1 (*LHD1*), or DTH8 a QTL for days to heading a TF that binds to the CCAAT-box and is a part of the HAP3 subunit (Wei et al. 2010; Thirumurugan et al. 2008; Laloum et al. 2012). When Hd5 is

introgressed, it delays flowering under LD by downregulating *Ehd1*, *Hd3a*, and *RFT1*, while under SD conditions the expression is not affected suggesting that flowering is independent of signaling cascade mediated by Ghd7 and Hd1 (Lin et al. 2003; Yan et al. 2011; Dai and Xue 2010). A cross between 'Nipponbare' (nonfunctional Hd6 allele due to a stop codon) and 'Kasalath' helped the identification of Heading date 6 (*Hd6*) that encodes for the α-subunit of CK2 and is a repressor of *Hd3a* and R*FT1* and hence responsible for delayed flowering (Ogiso-Tanaka et al. 2013; Takahashi et al. 2001). A key gene identified from a cross between 'Nipponbare' and 'Koshihikari' is Heading date 16 (*Hd16*)/Early flowering 1 (*EL1*) encoding a casein kinase I which is a repressor for flowering and is also presumed to be involved in the transition to flowering and between other developmental processes like tillering or hormonal signaling by phosphorylating *SLR1* the DELLA protein from rice and *Ghd7*. When deficient allele of Hd16 is introgressed into Nipponbare, it deteriorates photoperiodic sensitivity and induces the expression of *Ehd1*, *Hd3a*, and *RFT1*. Two MADS-box transcription factors, *OsMADS50* and *OsMADS56*, with antagonistic roles in flowering have also been identified. *OsMADS50* orthologous to *SOC1* (Suppressor of Overexpression of CO1) induces flowering by inhibiting *OsLFL1* that in turn is an inhibitor of *Ehd1* (putative B3 domain-containing transcription factor) (Lee et al. 2000; Peng et al. 2007; Peng et al. 2008). However, *OsMADS56* on the other hand when overexpressed in rice inhibits flowering by downregulating *Ehd1*, *Hd3a*, and *RFT1*. Comparative analysis of the two MADS transcription factors revealed the presence of K domains that is responsible for dimerization, and the C-terminus is shorter in *OsMADS56*. The presence of K-domain suggests that these two proteins are involved in complex formation and *OsMADS56* might be an inhibitor of *OsMADS50* (Cho et al. 1999; Lim et al. 2000; Li et al. 2009). Similarly, *OsDof12* was also identified as inducer of flowering (Li et al. 2009). Besides the regulation of the flowering under LD, there are several other factors that influence flowering under SD other than Hd1. These includes *OsMADS51*that when overexpressed in rice induces flowering by 10 days by triggering the expression of *Ehd1*, *Hd3a*, and *OsMADS14* but with no effect under LD. This leads to the hypothesis that *OsMADS51* might be an inducer/repressor for a SD-specific target protein. In addition, *OsCO3*, a negative regulator for flowering with similarity to *HvCO3* from barley belonging to CO-like proteins, has also been identified. The *OsCO3* overexpressed plants showed delayed flowering with reduced expression of *Hd3a* and *OsMADS14* (Kim et al. 2008; Park et al. 2008).

5 Constitutive Regulation of Flowering

Flowering is also regulated by constitutive factors like OsIndeterminate 1 (*OsId1*)/Early heading date 2 (*Ehd2*)/Rice Indeterminate 1 (*RID1*). Knockout or RNAi-mediated silencing of *OsId1*delayed flowering both under SD and LD conditions, respectively, thereby suggesting its essential role in flowering independent of light conditions (Matsubara et al. 2008; Wu et al. 2008). The probable mode of regulation by *OsId1* is that it directly interacts with *Ehd1* and hence its downstream genes Hd3a

and *RFT1*. Further, *OsID1* is orthologous to Id1 from maize suggesting that Id1–Ehd1 pathway is conserved in grasses (Galinat and Naylor 1951; Colasanti et al. 1998, 2006). Similar mode of regulation through the control of Ehd1 expression was observed in *ehd4* mutant (Early heading date 4) encoding a CCCH-type zinc finger protein. However, *OsCOL4* overexpression in rice resulted in delayed flowering both under LD and SD conditions with reduced expression of *Ehd1* suggesting its role as a negative regulator of flowering. In addition, the expression of *OsCOL4* is reduced in *osphyB* mutants suggesting that it is positively regulated by *OsPhyB* but through a different pathway as *OsCOL4* is insensitive to night break (NB) effect (Lee et al. 2010; Ishikawa et al. 2005). A bHLH transcription factor *OsLF* when overexpressed in rice leads to delayed flowering irrespective of photoperiodic conditions and partially reduced expression of *OsGI* and *Hd1*, hence indicating its role as a repressor. MicroRNAs like *miR172a* and *miR172d* are inducers of flowering through downregulation of AP2 transcription factors Supernumerary Bract (*SNB*) and *Oryza sativa* Indeterminate Spikelet1 (*OsIDS1*) and hence induced expression of *Ehd1*. These results suggest an essential role for miR172–AP2–Ehd1 pathway in controlling flowering (Aukerman and Sakai 2003; Choi et al. 2014).

6 Circadian Rhythm

CO (constans) the flowering time gene in *Arabidopsis* is controlled by GIGANTEA (*GI*), and this regulation is conserved in rice (Fowler et al. 1999). RNAi-mediated silencing of OsGI showed delayed flowering under SD but earlier under LD and also exhibited reduced sensitivity to photoperiod. Transcriptome analysis of WT and *osgi* mutants identified downregulation of several clock genes along with *Ehd1*, *Hd3a*, *Ghd7*, and *OsMADS51* suggesting that *OsGI*-mediated control occurs through several independent pathways (Izawa et al. 2011). Heading date 2 (Hd2) identified from QTL mapping using population derived from a cross between 'Nipponbare' and 'Kasalath' is closely linked to OsPRR37 (*Oryza sativa* Pseudo Response Regulator 37) that is a major component of circadian clock (Kaczorowski and Quail 2003; Yamamoto et al. 2003; Murakami et al. 2005; Farre and Kay 2007). OsPRR37 shows natural variation between different rice genotypes where some genotypes like 'Kitaake' and 'H143' have defective alleles (Kim et al. 2013; Koo et al. 2013). QTL mapping has also identified other flowering inducers like Heading date 3b (*Hd3b*)/Heading date 17 (*Hd17*)/Early flowering 7 (*Ef7*)/*Oryza sativa* Early flowering 3 (*OsELF3*). *OsELF3* is an ortholog of *Arabidopsis ELF3* gene and is diurnally regulated with highest expression during late night. Analysis of *OsELF3* mutant identified it as a negative regulator of *OsGI* and *Ghd7* under long-day conditions (Yang et al. 2013c). *OsELF3-1* and *OsELF3-2* orthologs of ELF3 from *Arabidopsis thaliana* have been identified to play an essential role in regulating photoperiodic flowering with *OsELF3-1* playing a more dominant role than the other. *OsELF3-1* positively regulates *OsLHY* while negatively regulates the expression of *OsPRR1*, *OsPRR37*, *OsPRR73*, and *OsPRR95*. Additionally, it is also involved in blue light signaling where it promotes flowering under short-day

conditions by inducing *Ehd1* and under long day by downregulating *Ghd7* (Zhao et al. 2012).

7 Photoreceptors and Flowering

Flowering in rice is influenced by photoreception, and *OsPhyA*, *OsPhyB*, and *OsPhyC* are important players. Researches carried out with *osphyB* and *osphyC* have identified them as repressors of flowering. Further, *OsPhyB* inhibits flowering by inducing the expression of OsCOL*4* and is also involved in the NB effect (Lee et al. 2010). Analysis of *osphyBosphyC* double mutants showed similar phenotypes as single mutants like early flowering under SD probably due to the involvement of posttranslational processing (Hirschfeld et al. 1998; Monte et al. 2003). In analogy with *Arabidopsis*, it can be concluded that *OsPhyB* and *OsPhyC* represses flowering by interacting to form a heterodimer, while they are unstable alone. Researches carried out with double mutants *osphyAosphyB* and *osphyAosphyC* showed early flowering under SD but delayed flowering under LD, while *osphyA* doesn't affect flowering. These observations clearly suggest that *OsPhyA* cooperates with other phytochromes for modulating the expression of regulators of flowering. Moreover, it has also been observed that *OsPhyA* induces the expression of *Ghd7*, while *OsPhyB* and *OsPhyC* are repressors. Though *OsPhyA* influences the expression of Ghd7, Ehd1 expression is not altered suggesting that *OsPhyA*-mediated regulation occurs via an alternate pathway. Photoperiod Sensitivity5 (*SE5*) gene coding for an enzyme involved in the biosynthesis of phytochrome was identified in rice from screening of γ-irradiated Bahia collection. The mutants exhibited early flowering and several other variations in the diurnal expression of genes involved in the photoperiod-mediated regulation of flowering and hence upregulation of *Hd3a* and *Ehd1*. These results thus suggest *SE5* as a negative regulator of flowering (Andrés et al. 2009). Analogous to *Arabidopsis* blue light receptor cryptochrome (*CRY*), rice has also three CRY genes *OsCRY1a*, *OsCRY1b*, and *OsCRY2*. *OsCRY2* antisense plants showed delayed flowering under both SD and LD identifying it as an inducer, but the exact mechanism is unknown. However, *OsCRY1a* and *OsCRY1b* play an important role in de-etiolation mediated by blue light (Hirose et al. 2006; Zhang et al. 2006). Os*HAL3* a highly conserved flavin mononucleotide (FMN)-binding protein when silenced in rice delayed flowering suggesting its role as an inducer. Further the changes in flowering time were not associated with altered expression of *Hd1* but through reduced accumulation of *Hd3a* and *MADS14*, and it also interacts with *Hd1* under dark. Thus, it can be concluded that *OsHAL3* positively influences flowering by forming a complex with *Hd1* (Su et al. 2016).

8 Epigenetics: Chromatin Modeling, Histone Methylation, and Demethylation

Flowering genes are regulated developmentally. As has been observed for *OsLFL1* (*Oryza sativa* LEC2 and FUSCA3 Like 1), its transcript accumulation decreases eventually with plant maturity. Contrary to the expression of OsLFL1, the expression level of *OsVIL2* (*Oryza sativa* VIN3-like 2)/*LC2* (Leaf inclination 2)/*OsVIL3* (*Oryza sativa* VIN3-like 3)) increases with decreased expression of the aforementioned. Overexpression of *OsLFL1* and knockout of *OsVIL2* delayed flowering both under SD and LD (Yang et al. 2013a; Wang et al. 2013). Interaction of *OsVIL2* with histone H3 and *OsLFL1* chromatin has been proved in osvil2 mutants. H3K27me3 levels of *OsLFL1* chromatin was observed. This observation proves that *OsVIL2* is a repressor of *OsLFL1* (Yang et al. 2013b). In other researches it has been observed that *OsVIL2* also interacts with *OsEMF2b* (*O. sativa Embryonic Flower 2b*) that forms the core for polycomb repressive complex 2 (*PRC2*) and in turn is a repressor for downstream genes (Yang et al. 2013b). Analysis of *osemf2b* mutants has identified *OsEMF2b* as an activator of flowering by downregulating the expression of *OsLFL1* (Conrad et al. 2014; Xie et al. 2015). Similar phenotypes of delayed flowering were observed in RNAi plants of *OSVIL1*. It was postulated that *OsVIL1-OsVIL2* interacts to promote flowering. *OsTrx1* (*Oryza sativa* Trithorax 1) encodes a histone methyltransferase that controls the expression of downstream target genes by modifying the chromatin. Delayed flowering under LD conditions were observed in ostrx1 and ehd3 mutants with increased level of Ghd7 suggesting that *Ghd7* is negatively regulated by cooperative interaction of *Ehd3* and *OsTrx1* (Choi et al. 2014; Matsubara et al. 2011). Besides, there are SET domain group (SDG) proteins which may be involved in remodeling of chromatin by histone demethylation (Ng et al. 2007). Researches carried out with a histone methyltransferase, *lvp1* (Long Vegetative Phase 1) mutant, showed reduced expression of *OsMADS50*, *Hd3a*, *RFT1*, and *Ehd1* and delayed flowering under LD. These observations identified *LVP1* as a selective flowering inducer under LD by regulating the expression of OsMADS50 (Sun et al. 2012). In addition, analysis of RNAi plants for *SDG711* and *SDG718* encoding the zeste [E(z)] subunit of *PRC2* displayed early and delayed flowering under LD and SD conditions, respectively. These results identified them as a LD-/SD-specific flowering repressor and activator (Liu et al. 2014). Lysine Specific Demethylase 1 (*LSD1*) and Jumonji C (jmjC) domain-containing proteins are histone demethylases that can eliminate histone methylation (Shi et al. 2015). *LSD1* is an amine oxidase that is dependent on flavin and is involved in regulation of flowering (Shafiq et al. 2014). Os02g0755200, Os04g0560300, and Os08g0143400 encoding *LSD1* homologs in rice have been identified but are not characterized. Moreover, there are jmjC domain-containing proteins like *JMJ706* that demethylates H3K9me2/me3 are also known in rice. *JMJ705*, another histone lysine demethylase, removes H3K27me2/3 and is also induced by abiotic stress and pathogen attack (Li et al. 2013). Histone acetylations and H3K4 and H3K36 methylations have been proposed to be a key player that are involved in the expression of several genes involved in rice flowering. When

OsHDT1 a member of the HD2 family is overexpressed in rice, it led to early flowering under LD by downregulating *OsGI* and *Hd1* (Li et al. 2011a). S-Adenosyl-L-methionine synthetase is essential for methylation of DNA and protein, and its deficiency led to late flowering with reduced methylations of H3K4me3 and DNA CG/CHG and downregulation of Ehd1, Hd3a, and RFT1 (Li et al. 2011b). Similarly, analysis of JmjC-domain encoding protein *Se14* (Photoperiod sensitivity-14) mutant revealed increase expression of *RFT1* due to elevation of H3K4me3 under LD conditions (Yokoo et al. 2014). Researches carried out later flowering mutant of *SDG725* have identified H3K36-methyltransferase (*SDG725*) at the upstream of H3K36 which when methylated deposits H3K36me2/me3 at the chromatin regions of genes involved in different signaling cascades and biosynthesis of brassinosteroids (Sui et al. 2012, 2013).

9 Environmental Cues and Flowering

Though several abiotic and biotic stresses impact flowering, stress-regulated flowering is not considered as a mechanism controlling floral transition. Researches over the years have shown that they are an essential factor that needs to be considered. When rice is exposed to drought at its growing stage, flower development gets stalled and hence leads to sterility. Recently, it has been shown that drought stress significantly affects the reproductive stage, but the plants have a strong recovery capacity that can be a point of interest for breeders (Yang et al. 2019). In an effort to get out of drought stress, floral development gets accelerated triggering drought escape. A similar concept that needs to be considered is known as drought avoidance where the plant reduces water consumption to minimize the effect of stress. It has been observed in *Arabidopsis* that when exposed to drought stress under long or short days, the flowering time gets accelerated or delayed, respectively. Analysis of *GI* (Gigantea) has shown that it facilitates drought escape by downregulating CDF (Cycling Dof Factor) that represses the inducers of flowering CO and FT (Sawa and Kay 2011). Similarly, in rice *Ghd7* (Grain Number, Plant Height and Heading Date 7) that is a negative regulator for heading under long days is also a inducer for drought sensitivity (Weng et al. 2014). When *OsFTL10* was overexpressed in rice, it not only induced flowering by interacting with 14-3-3 and *OsFD1* but also conferred drought tolerance (Fang et al. 2019). Similar results were observed on overexpressing OsWOX13, but it may be a regulator for drought escape (Minh-Thu et al. 2018). Under the face of current imminent threat of global climate change, temperature extremes also impact flowering and hence yield and quality in rice (Zhang et al. 2018). QTL mapping carried out with BC5F2 population derived from IR64/N22 identified qHTSF4.1 that not only increased spikelet fertility but also enhanced heat tolerance (Ye et al. 2015). qEMF3 a QTL for early morning flowering was identified from a NIL population developed from wild rice; *O. officinalis* (CC genome) showed that plants can escape the adverse effects of heat stress by advancing flowering time (Hirabayashi et al. 2014). Similar results for enhanced cold tolerance at reproductive stage was observed with improved pollen seed fertility

on overexpressing bZIP73^{Jap} conferred through bZIP71-bZIP73^{Jap} -qLTG3-1^{Nip} sugar transport pathway (Liu et al. 2019). To conclude, it can be said that it is essential to make use of the natural diversity in rice to develop climate resilient rice that can withstand stress and flower and yield favorably.

10 Conclusions

A signal transduction cascade mediated by *GI–Hd1–Hd3a* has been found to be conserved in all plant species. However, rice has some other pathways that are coupled with *Ehd1* or are linked directly to different florigens. Two such important pathways under LD are mediated by *Ghd7* and *OsMADS50* that is regulated not only by phytochromes but also by chromatin remodeling. Under short-day light conditions, *OsMADS51* and *OsCO3* are inducers and inhibitors of flowering. *OsID1* and *OsCOL4* are inducers and repressors of flowering. miR172 induces flowering by degrading the mRNA from AP2 transcription factors. A summary of different methods of regulation of flowering in rice is shown in Fig. 1. In spite of researches over the years that have led to the identification of several regulators, their interrelationships and signaling cross talk is not well understood and needs more attention. Apart from the factors mentioned, there are several other factors like (1) responses arising from exposure to environmental stress or (2) nutrient deficiencies that needs to be considered for manipulating the flowering process to improve yield potential.

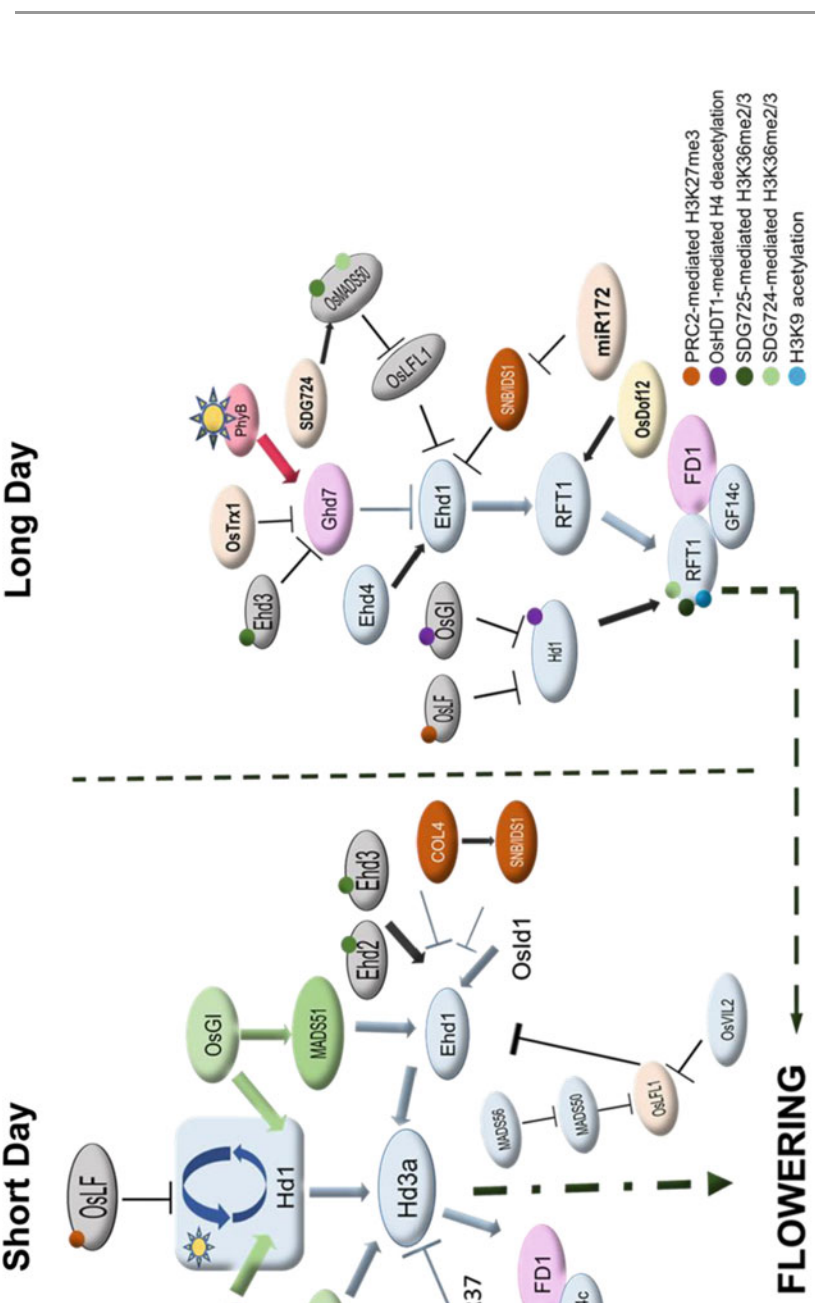

Fig. 1 Regulatory network of genetic and epigenetic control of flowering in rice under short-day and long-day conditions. Flowering in rice is mediated by two florigens Hd3a and RFT that are in turn regulated by Hd1- or Eh1-dependent pathways. The expressions of Ehd1 and hd1 are regulated by different genes that are shown in the figure in different circles. The arrows represent activation, while the bar indicates downregulation. The circles at the top of some of the flowering genes indicate different modifications inflicted by histone modification. All the information about genes represented in the figure are collected from literature search

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Breeding and Bioengineering of Male Sterility in Rice

K. N. Poornima, S. J. Satheesh Naik, and Abhishek Bohra

Abstract

Male sterility has been an important trait for heterosis-based breeding programs. Harnessing hybrid vigor has been a promising approach to tackle the current challenges of increased population and climate change. Recent advancements in understanding the molecular basis of male sterility phenomenon have helped breeders develop desired varieties. The molecular approaches for the generation of male-sterile lines through regulation of phytohormonal biosynthesis in reproductive organs are also under way. The era of omics have enriched the understanding of genes cytoplasm and nuclear communication, and further molecular tools such as DNA markers implicated in hybrid breeding have facilitated further understanding of their interactions. Bioengineering is the application of engineering principles to the fields of biology and health care. Bioengineering of crop plants for improved tetrahydrofolate production is one such example. There are three types of male sterilities, viz., nuclear male sterility (NM), cytoplasmic male sterility (CMS), and the other cytoplasmic genetic male sterility (CGMS). CMS genes may be formed during evolution through gene duplication and multi-recombination, and the corresponding nuclear Rf genes appear to have undergone coevolution. The mitochondrion being a semiautonomous organelle with its own genome encodes genes that control TCA cycle and ATP generation. Until now 28 CMS genes have been identified from 13 crop species. The knowledge obtained about the molecular mechanisms of these nuclear and cytoplasmic genes can be used in hybrid development using bioengineering. Bioengineering in plants such as development of golden rice has been successful as a technology, but it has been facing challenges with respect to its acceptance among the public due to safety issues that need to be resolved at the earliest.

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Keywords

CMS-Cytoplasmic Male Sterility · NM-Nuclear Male Sterility · CGMS-Cytoplasmic Genetic Male Sterility · Breeding

1 Introduction

Plant breeding is a fascinating science of developing novel plant varieties with improved grain yield, quality, or nutritional status. Human selection has been imposed on multigeneration on a genetically diverse population to obtain plants of desirable genotypes. The selection process is driven by biological assessment in relevant target environments, regions of plants, and knowledge of genes and genomes. Biomedical engineering, or bioengineering, is the application of engineering principles to the fields of biology and health care. Bioengineers work with biologists to develop systems, equipment, and devices in order to solve clinical problems. Bioengineering of crop plants for improved tetrahydrofolate production by Chaudhary et al. (2018) is one such example. Male sterility in plants is the failure of plants to produce functional anthers, pollen, or male gametes. There are two types of it: one is cytoplasmic male sterility (CMS), and the other is cytoplasmic genetic male sterility (CGMS). CMS genes may be formed during evolution through gene duplication and multi-recombination, and the corresponding nuclear Rf genes appear to have undergone coevolution. The mitochondrion being a semiautonomous organelle with its own genome encodes genes that control TCA cycle and ATP generation. The reported spatiotemporal specific accumulation of cytotoxic CMS proteins in tapetum finally leading to PCD (programmed cell death) in sporophytic or gametophytic cells leads to male sterility. Until now 28 CMS genes have been identified from 13 crop species. Deployment of genome-scale techniques like GWAS will help illuminate the genetic landscape of fertility restoration trait with enhanced resolution (Feng et al. 2015). Further, as demonstrated recently, wheat implementation of genome-based predictions for improving hybrid performance could witness notable achievements such as sustained gains resulting from heterotic group construction (Zhao et al. 2015), and the genomic selection becomes particularly relevant in case of relatively low or "realistic" prediction accuracies (Longin et al. 2015).

2 Discovery of CMS and Its Importance

CMS has been one of the great gifts of nature that has been harnessed to develop the most fruitful hybrid systems in crop plants. Male sterility is a condition wherein the male reproductive parts of the plant are either absent, aborted, or nonfunctional and hence incapable of natural sexual reproduction. The situation may arise at any time during any developmental defect at the stage of microsporogenesis or at release of pollen grains. Individuals in some natural population with these malformations in pollen were first found by Kolreuter in 1763. Later Darwin recognized in 1877 that

the loss of reproducing ability in plants helps evolutionary process in enhancing adaptation through gene transfer by cross-pollination from related and unrelated individuals. As far as male sterility in crop breeding is concerned, individuals with altered male fertility keep their female fertile so that they may be fertilized from another individual and produce viable seed. Lack of knowledge of the use of these male sterile systems has led to loss of some of the novel lines over a period of time. The establishment of the concept of heterosis by Shull (1908) and thereafter by others has led to development of crops through hybrids. The first hybrid seed development was in sorghum by Stephens in 1937, and during the same time period, hybrid seed production of onion also took place. The male sterile phenotype is expressed either through natural mutation, induced mutagenesis, or through hybridization and selection processes. Hybrid plant breeding is an upscaled technology wherein hybrid vigor or heterosis is harnessed for crop improvement. About 150 plant species have been reported to have CMS system induced through mutagenesis wide/interspecific hybridization, protoplasmic fusion genetic engineering, or existing in nature (Yamagishi and Bhat 2014; Wang et al. 2013; Singh et al. 2015).

3 Fundamentals of Male Sterility Systems

Male sterility is the defect in higher plants that arise due to inability of anther tissues to grow and differentiate normally, failure of normal microsporogenesis, failure to release the mature pollen grains, and/or inability of mature pollen grains to germinate on the stigmatic surface. However these changes do not impair the female reproductive system and such plants when pollinated through manual or natural means they produce seeds. Since male sterility is a manifestation of abnormal genotype, it may vary across crops and genotypes.

4 Morphological Changes Associated with Male Sterility

The morphological changes that precede CMS in the various systems occur at different developmental stages and in different tissues. Nevertheless, in a number of systems, one of the first visible signs of CMS is the premature degeneration of the tapetum layer of the anther that leads to production of nonfunctional pollens. Ultimately the plants, which do not have out-crossing behavior, end up without producing seed.

In T-cytoplasm maize, the first obvious morphological lesion occurs soon after meiosis, when the mitochondria of the tapetum and middle layer of the anther begin to degenerate 10. PET1-mediated male sterility in sunflower is also associated with a degeneration of the tapetum and tetrads after meiosis II. However, in this system, differences between fertile and sterile plants first become visible as early as the beginning of meiosis. Similarly, in petunia with CMS, cells of the tapetal layer become vacuolated during meiosis 11.

5 Types of Sterility in Plants

Nuclear Male Sterility (NMS) Genetic male sterility also called as Nuclear Male Sterility (NMS) is the most common form of male sterility system found in both monocots and dicots (Kaul 1988). Spontaneous mutation is a common source in nature for nuclear male sterility. As pointed out by Duvick (1966), NMS can probably be found in all diploid species. Mutations resulting in nuclear male sterility have also been induced in several important crop plants. Spontaneous nuclear male sterility is usually controlled by a single recessive gene. This does not permit the creation of a population where all plants are male sterile. The highest proportion of male steriles that can be realized is 50%, which is obtained in the backcross with genotype asmsms × MSms. Less frequently, nuclear male sterility has been found to be controlled by more than one recessive gene (Driscoll, 1986), by polygenes (Athwal et al. 1967) or by dominant genes (Mathias et al. 1985). Both ionizing radiation and chemical mutagens such as ethyl methane sulfonate (EMS) and ethylene imine (EI) have been used in species like maize, tomato, and barley, which have been intensively studied, more than 40 non-allelic male sterility genes by Kaul (1988).

Genetic male sterility is controlled by nuclear genetic factors, independent of cytoplasmic influences. Its expression is controlled by one or two pairs of recessive alleles which segregate independently. An exception of existence of lines controlled by one or two dominant alleles has also been reported. Phenotyping becomes easy when there is a linkage between male sterile gene and any of the morphological trait such as pigmentation of the stem, delayed flowering, sparse podding, translucent anthers, etc. (Kaul 1988; Verulkar et al. 1997).

Cytoplasmic Male Sterility (CMS) CMS is found to be governed by defective mitochondrial genome arising through deleterious interactions of mitochondrial genes with those present in the nucleus. Such a cytoplasm is referred to as sterile and can originate spontaneously or through wide hybridization. Based on CMS origin, they are classified into autoplasmic CMS and alloplasmic CMS. Autoplasmic CMS would then refer to MS arisen within a species as a result of spontaneous mitochondrial genomic mutations. Alloplasmic on the other hand arisen from inter-generic, interspecific, or occasionally intraspecific crosses and where the MS can be interpreted as being due to incompatibility of poor cooperation between nuclear genome of one species and organellar genome of another. The pollen grains of MS plants are not functional as their nucleus also contains a pair of recessive non-restoring alleles. The fertile or normal cytoplasm and non-restoring recessive nuclear alleles maintain the CMS nature of the genotype. The conclusive evidence, linking CMS to mitochondrial genome, still exists to considerable species of plants. Nevertheless, it cannot be excluded that occasionally CMS is related to chloroplast DNA that at least contributes to CMS in *Nicotiana* (Frankel et al. 1979), *Gossypium* (Galau and Wilkins 1989), and *Sorghum* (Chen et al. 1990).

Cytoplasmic Nuclear Male Sterility (CGMS) This system of MS appears due to interaction between nuclear and cytoplasmic genomes. The difference between CMS and CGMS lies in the fertility restoration mechanism of the individual. At the molecular level, this kind of MS is expressed due to rearrangement of the mitochondrial genome which results in production of toxic proteins, and reduction of respiration gets corrected by the restorer genes residing in the nucleus. This complementation leads to the development of CMS line and its corresponding maintainer line and restorer line. According to Iwabuchi et al. (1993), the abnormal mitochondrial gene produced an additional ORF with the encoded mRNA. Alterations in the promoter regions and portions of the coding regions of the mitochondrial ATP synthase also led to MS as reported by Hanson and Bentolila (2004).

6 Methods of Producing Male Sterile Plants

Since the first transgenic male sterility system was described, many different strategies to produce male sterile plants by interfering with the development and the metabolism of the tapetum (Mariani et al. 1990; van der Meer et al. 1992; Kriete et al. 1996; Hernould et al. 1998) or pollen (Worrall et al. 1992) in transgenic plants have been reported. Male sterility was further induced by using sense or antisense suppression to inhibit essential genes (Xu et al. 1995; Luo et al. 2001) or by expressing aberrant mitochondrial gene products (Hernould et al. 1993; He et al. 1996; Gómez-Casati and Iglesias 2002).

However, any of the available strategies has drawbacks such as interference with metabolism or general development or restriction to specific species. Thus, a universal and dominant male sterility system with efficient effect on pollen growth offering the possibility to efficiently restore fertility would be a great advantage for the production of hybrid seeds.

7 Identification and Characterization of Sterility-Associated Genes in Crop Plants

It was initially assumed that CMS is the result of lesions in either the mitochondrial or the chloroplast genomes by virtue of its maternal inheritance. Indeed, in all cases where a specific CMS associated gene has been identified and shown via correlative or direct means to be responsible for CMS, the lesion has been in the mitochondrial genome. However, because plant mitochondrial genomes are large (200–2400 kb), it is often difficult to identify the sequences responsible for CMS. Several approaches have been used to narrow the search, including comparative physical mapping and the identification of differences in mitochondrial gene-expression patterns between normal fertile, male sterile, restored fertile, and fertile revertant plants. The key test, a functional assay for a candidate sequence, has been reported in PET1 cytoplasm of

sunflower, T- and S-cytoplasms of maize, Bo cytoplasm of rice, and A3 cytoplasm of sorghum.

CMS-associated genes are often chimeric, having been derived from fusions of portions of known genes with previously unknown sequences. In most instances the sequences of CMS-associated ORFs are unrelated. However, many of these CMS-associated genes encode large hydrophobic domains as a common feature. There are only two examples of CMS-associated genes that exhibit sequence similarity. In *B. napus*, orf222 from the nap cytoplasm is 79% similar at the amino acid level to orf224 from the pol cytoplasm. orf107 of the A3 cytoplasm of sorghum and the 3′-region of orf79 from rice comprise the only pair of interspecific CMS-associated ORFs known to exhibit a high level of sequence similarity.

The answer to how CMS-associated genes cause male sterility has arisen in several systems. For example, in *Phaseolus* the ORF239 protein is associated with the cell walls of developing microspores. A similar association is observed in transgenic tobacco expressing nuclear copies of orf239 (He et al. 1996). These transgenic plants are also male sterile when the transgene lacks a mitochondrial targeting sequence. Thus, it can be concluded that the ORF239 protein is toxic to developing microspores regardless of where it is encoded or translated. These results suggest that CMS in *Phaseolus* is not directly related to mitochondrial function.

In addition to being male sterile, maize that carries T-cytoplasm is highly sensitive to the host-selective toxin (T-toxin) produced by race T of *Cochliobolus heterostrophus* Drechsler (asexual stage *Bipolaris maydis* Nisikado and Miyake), the causal organism of southern corn leaf blight. By contrast, tissue culture-derived male fertile mutants of T-cytoplasm that have lost the T-urf13 gene are toxin insensitive. This finding suggests that T-urf13 is responsible for both the male sterility and toxin sensitivity. Indeed, the URF13 protein confers toxin sensitivity when expressed in *E. coli*, yeast, or tobacco. However, none of the toxin-sensitive, transgenic tobacco plants produced to date has been male sterile even when URF13 has been targeted to the mitochondria. Similarly, expression of the PCF protein (which is associated with CMS in petunia) has not caused male sterility in transgenic tobacco or petunia produced to date. The failure to obtain male-sterile plants from these two experiments suggests that correct tissue-specific expression and to cause male sterility subcellular localization are required for some CMS-associated proteins.

Detailed RFLP analyses revealed that the PET1 cytoplasm of sunflower differs from fertile cytoplasms (including that of *H. petiolaris*) within a 17-kb region of the mitochondrial genome that includes a 12-kb inversion and a 5-kb insertion flanked by 261-bp inverted repeats. These polymorphisms relative to the cytoplasm of *H. petiolaris* are particularly interesting given that the PET1 cytoplasm is derived from an interspecific hybridization of *H.petiolaris* and *H. annuus*. The 5-kb insertion created a 522-bp open reading frame (ORF) downstream of the atpA gene 12. This ORF encodes a 16-kDa protein that accumulates in both sterile and restored PET1 seedlings (Horn et al. 1991). Accumulation of the orfH522 transcript and its encoded protein exhibits tissue-specific (Monéger et al. 1994) and cell-specific (Smart et al.

1994) reductions following restoration. These results therefore provide strong support for the role of orfH522 in CMS.

In *B. napus*, there are several CMS-associated ORFs associated with male sterility. Physical mapping has revealed differences between the male-sterile pol and male-fertile cam mitochondrial genomes that are confined to a rearranged region around the atp6 gene (L'Homme et al. 1993). This result led to the discovery of the CMS-associated orf224/atp6 locus, which is present only in the pol cytoplasm. Additional evidence for the involvement of this locus in the CMS phenotype is that orf224/atp6 transcripts are differentially processed in plants that carry the Rfp1 or Rfp2 restorers (Singh et al. 1996). Interestingly, the predicted ORF224 protein exhibits 79% sequence similarity to the predicted protein of the orf222 portion of the CMS-associated orf222/nad5c/orf139 region in the male-sterile nap cytoplasm (L'Homme et al. 1997). The basis for calling this region CMS-associated is that orf222/nad5c/orf139 transcripts are less abundant and are qualitatively different in restored and non-restored (i.e., sterile) plants. Again, based on physical mapping studies, orf138 is the best candidate as the CMS-associated gene in the male-sterile ogu cytoplasm (Bonhomme et al. 1992). Unlike the CMS-associated genes in the pol and nap cytoplasms of canola, however, the accumulation of orf138-specific transcripts is not correlated with the presence or absence of the Rfo restorer. Nevertheless, restoration does appear to be associated with a reduced accumulation of the 19-kDa ORF138 mitochondrial-membrane protein (Grelon et al. 1994).

In maize, there are numerous RFLPs between the mitochondrial genomes of the male-fertile (N-) and male-sterile T-cytoplasms. This greatly complicated the search for the gene responsible for sterility in the mitochondrial genome. The T-urf13 gene was ultimately identified via two complementary approaches (Levings 1993). A mitochondrial DNA fragment that hybridized to a family of T-specific transcripts was seen in one approach. Sequence analysis of this T-specific region revealed two ORFs, T-urf13 and the co-transcribed orf221 (Dewey et al. 1986). Alterations in T-urf13-specific transcript accumulation occur in plants restored to fertility by the Rf1 gene, and this alteration is absent in plants carrying mutant rf1-m alleles. Recently, it has been revealed that the Rf8 and Rf∗ partial restorers also have specific effects (distinct from Rf1) on T-urf13 transcript accumulation (Dill et al. 1997). The second approach took advantage of tissue culture-induced mitochondrial mutations that restored fertility to T-cytoplasm. Physical mapping experiments revealed a common RFLP in 19 out of 20 such mutants. Subsequent analyses revealed that the T-urf13 reading frame had been lost by homologous recombination in each of the mutants. The remaining fertile mutant, T-4, which did not exhibit the RFLP, contained a 5-bp insertion in theT-urf13 coding region that truncated this reading frame. In all of these mutants, the co-transcribed orf221 reading frame is unaltered. T-urf13 encodes a 13-kDa mitochondrial pore-forming protein (URF13) that assembles as an oligomer in the inner mitochondrial membrane. The accumulation of this protein is reduced in plants restored to fertility by Rf1 (Dewey et al. 1987) or Rf8 (Dill et al. 1997). Hence, in combination, these approaches provide strong evidence that the T-urf13 gene is responsible for CMS in T-cytoplasm maize. The mitochondrial genome of the male sterile S-cytoplasm of maize contains the repeated

DNA region 'R,' which contains two chimeric ORFs (Zabala et al. 1997). This region is rearranged in fertile revertants of S-cytoplasm plants. In addition, the nuclear restorer Rf3 alters the abundance of the major R transcripts. More specifically, an R-derived 1.6-kb transcript that accumulates in sterile plants is absent in fertile revertants and reduced in plants restored to fertility by Rf3.

8 Male Sterility System in Rice

The first type of male sterility used in hybrid rice breeding constitute the wild-abortive CMS (CMS-WA), and it has been the most important approach in developing number of hybrids and the total area planted to those hybrids. Genetic vulnerability of hybrid rice arose, and insufficient genetic diversity was considered to be a major reason why rice yields plateaued (Cheng and Cheng 2000). During the previous decade, great attention had been paid to broaden the genetic diversity of hybrid rice.

The use of indica × japonica crosses has long been considered a promising approach to broadening the genetic diversity and enhancing the heterosis of rice. However, F_1 semi-sterility has generally been encountered in inter-subspecies crosses of rice, making it meaningless for direct use in hybrid rice breeding. In addition, distant crosses do not always increase F_1 yield, and this is particularly true when the parental lines belong to different subspecies (Zhang et al. 1996; Xiao et al. 1996).

CMS is a maternally inherited trait and is often associated with unusual open reading frames (ORFs) found in mitochondrial genomes, and in many instances, male fertility can be restored specifically by nuclear-encoded, fertility restorer (Rf) genes (Schnable and Wise 1998).

Gibberellic acid (GA) is a very important phytohormone which regulates different aspects of plant development that includes embryo development, stem elongation, and flower development particularly in anthers. GA-deficient mutants were observed to have abnormal anther developments which led to male sterility. The tapetum plays a crucial role in pollen development. This secretory tissue produces numerous nutritive proteins necessary for pollen maturation. The tapetum, whose cells undergo programmed cell death (PCD), is completely diminished by the time the pollen is fully mature. Previous studies on a thermosensitive genic male-sterile (TGMS) rice (*Oryza sativa* L.) suggested that failure in pollen development led to male sterility. Later it was described that male sterility is associated with premature PCD of the tapetum.

Cytological observations of TGMS rice anthers at various developmental stages indicated that PCD initiates at an early stage of pollen development and continues until the tapetal cells are completely degraded, resulting in pollen collapse. Transmission electron microscopy showed the morphologically distinct hallmarks of apoptosis, including cytoplasmic shrinkage, membrane blebbing, and vacuolation. Identification of DNA fragmentation using the TUNEL assay supports the hypothesis that premature PCD is associated with male sterility in the rice. The

tissue-specific feature of the thermosensitive genic male-sterile phenotype is discussed with regard to PCD during anther development (Ku et al. 2003).

Cytological study of male sterility has demonstrated that developmental defects occurring during microgametogenesis cause pollen abortion in rice (Laser and Lersten 1972). CMS and nucleus-controlled fertility restoration are widespread plant reproductive features that provide useful tools to exploit heterosis in crops. However, the molecular mechanism underlying this kind of cytoplasmic–nuclear interaction remains unclear. It has been shown in rice (*Oryza sativa*) with Boro II cytoplasm that an abnormal mitochondrial open reading frame, orf79, is co-transcribed with a duplicated atp6 (B-atp6) gene and encodes a cytotoxic peptide. Expression of orf79 in CMS lines and transgenic rice plants caused gametophytic male sterility. Immunoblot analysis showed that the ORF79 protein accumulates specifically in microspores. Two fertility restorer genes, Rf1a and Rf1b, were identified at the classical locus Rf-1 as members of a multigene cluster that encode pentatricopeptide repeat proteins. RF1A and RF1B are both targeted to mitochondria and can restore male fertility by blocking ORF79 production via endonucleolytic cleavage (RF1A) or degradation (RF1B) of dicistronic B-atp6/orf79 mRNA. In the presence of both restorers, RF1A was epistatic over RF1B in the mRNA processing. It has also been shown that RF1A plays an additional role in promoting the editing of atp6 mRNAs, independent of its cleavage function (Wang et al. 2003).

Sterility is common in hybrids between divergent populations, such as the indica and japonica subspecies of Asian cultivated rice (*Oryza sativa*). Although multiple loci for plant hybrid sterility have been identified, it remains unknown how alleles of the loci interact at the molecular level. Photoperiod- and thermosensitive genic male sterility (PGMS and TGMS) are the core components for hybrid breeding in crops. Hybrid rice based on the two-line system using PGMS and TGMS lines has been successfully developed and applied widely in agriculture. One of the studies showed that a locus for indica-japonica hybrid male sterility, Sa, comprises two adjacent genes, SaM and SaF, encoding a small ubiquitin-like modifier E3 ligase-like protein and an F-box protein, respectively. Most indica cultivars contain a haplotype $SaM^{+}SaF^{+}$, whereas all japonica cultivars have $SaM^{-}SaF^{-}$ that diverged by nucleotide variations in wild rice. Male semi-sterility in this heterozygous complex locus is caused by abortion of pollen carrying SaM^{-}. This allele-specific gamete elimination results from a selective interaction of SaF^{+} with SaM^{-}, a truncated protein, but not with SaM^{+} because of the presence of an inhibitory domain, although SaM^{+} is required for this male sterility. Lack of any one of the three alleles in recombinant plants does not produce male sterility. It can be said that a two-gene/three-component interaction model is present for this hybrid male sterility system. The findings have implications for overcoming male sterility in inter-subspecific hybrid rice breeding (Long et al. 2008).

Plant male reproductive development is highly organized and sensitive to various environmental stressors, including high temperature. Global warming may increase the instability of rice yields even in temperate regions, mainly due to the increased probability of male sterility induced by high temperatures (Horie et al. 1996). High or low temperature stress results in a lower seed set due to male sterility in most

crops, including tomatoes (Peet et al. 1998; Sato et al. 2002), cowpeas (Ahmed et al. 2000), wheat (Saini and Aspinall 1982), barley (Sakata et al. 2000; Oshino et al. 2011), and rice (Satake and Yoshida 1978; Prasad and Snyder 2006). It has been reported that high temperatures on the day of flowering decrease the ability of pollen grains to swell, thus resulting in poor anther dehiscence (Matsui et al. 2000). However, little is known about how high temperatures at the microspore stage induce sterility in rice (Endo et al. 2009). This finding was consistent with a previous study reporting that reduction of spikelet fertility in rice was caused by high temperatures during the booting stage, 9 days before heading (Satake and Yoshida 1978). Nuclear restorer (Rf or Fr) genes function to suppress the deleterious effects of CMS-associated mitochondrial abnormalities by diverse mechanisms. There are now several well-characterized CMS systems, for which the mitochondrial sequences thought to be responsible have been described.

Hybrid rice has greatly contributed to the global increase of rice productivity. A major component that facilitated the development of hybrids was a mutant showing photoperiod-sensitive male sterility (PSMS) with its fertility regulated by day length. Transcriptome studies have shown that large portions of the eukaryotic genomic sequences are transcribed to long noncoding RNAs (lncRNAs). However, the potential roles for only a few lncRNAs have been brought to light at present. Thus, great efforts have to be invested to understand the biological functions of lncRNAs (Ding et al. 2012). For hybrid rice breeding, PSMS rice can be used to propagate itself under short days and to produce hybrid seeds by interplanting it with normal fertile lines under long-day conditions. Moreover, PSMS rice has a broad spectrum of restoration; all of the normal rice varieties tested can restore the fertility of F1 hybrids (Li et al. 1997). Two-line hybrids developed using this PSMS germplasm have occupied millions of hectares of rice fields mostly in China for more than a decade. PSMS germplasms have also been found and explored in several other crops. It has been shown that a locus regulating PSMS in rice encodes a lncRNA. A SNP between 58 N and 58S in this lncRNA caused epigenetic modifications, which reduced expression of this lncRNA, resulting in male sterility under long-day conditions (Ding et al. 2012).

However, the molecular mechanism underlying the control of PGMS and TGMS remains obscure. In this study, we mapped and cloned a major locus, p/tms12–1 (photo- or thermo-sensitive genic male sterility locus on chromosome 12), which confers PGMS in the japonica rice line Nongken 58S (NK58S) and TGMS in the indica rice line Peiai 64S (PA64S, derived from NK58S). A 2.4-kb DNA fragment containing the wild-type allele P/TMS12–1 was able to restore the pollen fertility of NK58S and PA64S plants in genetic complementation. P/TMS12–1 encodes a unique noncoding RNA, which produces a 21-nucleotide small RNA that was named osa-smR5864w. A substitution of C-to-G in p/tms12–1, the only polymorphism relative to P/TMS12–1, is present in the mutant small RNA, namely, osa-smR5864m. Furthermore, overexpression of a 375-bp sequence of P/TMS12–1 in transgenic NK58S and PA64S plants also produced osa-smR5864w and restored pollen fertility. The small RNA was expressed preferentially in young panicles, but its expression was not markedly affected by different

day lengths or temperatures. Further results revealed that the point mutation in p/tms12–1, which probably leads to a loss-of-function for osa-smR5864m, constitutes a common cause for PGMS and TGMS in the japonica and indica lines, respectively. The above findings thus suggest that this noncoding small RNA gene is an important regulator of male development controlled by cross-talk between the genetic networks and environmental conditions (Zhao et al. 2012).

Yet in another study, it was found that mutation of thermosensitive genic male sterile 5 (tms5) in rice causes the TGMS trait through a loss of RNase Z^{S1} function. They showed that RNase Z^{S1} processes the mRNAs of three ubiquitin fusion ribosomal protein L40 (Ub_{L40}) genes into multiple fragments in vitro and in vivo. In tms5 mutants, high temperature results in increased levels of Ub_{L40} mRNAs. Overaccumulation of Ub_{L40}mRNAs causes defective pollen production and male sterility. The study uncovers a novel mechanism of RNase Z^{S1}-mediated Ub_{L40} mRNA regulation and shows that loss of this regulation produces TGMS in rice, a finding with potential applications in hybrid crop breeding (Zhao et al. 2014).

New discoveries have been continuously made in recent years on the roles of noncoding RNAs in regulating biological processes. Phased small-interfering RNAs (phasiRNAs) may be the newest member discovered in recent years. The photoperiod-sensitive male sterility (PSMS) rice is a very valuable germplasm that started the era of two-line hybrid rice. One of the research results show that phasiRNAs generated by a long-noncoding RNA PMS1T encoded by the Pms1 locus regulates PSMS in rice. This work provides a case associating the phasiRNAs with a biological trait, especially an agriculturally highly important trait, thus confirming that the phasiRNAs indeed have biological functions (Fan et al. 2016).

9 Conditioned Reversible Male Sterility for Production of Female Seed

One approach to multiply male sterile plants is to produce plants that are conditionally fertile. During female parent multiplication, male sterile plants are treated with a fertility-restoring chemical and can be self-fertilized. This system has some advantages over the selection of male sterile plants by herbicide application, for example, that the chemical has to be used during female parent multiplication and not during hybrid seed production and it can be applied to a smaller acreage. Based on conditional male fertility, several pollination control systems have been described. An example for the regulation of male fertility is the manipulation of hormones in male reproductive tissues. Huang et al. (2003) induced male-sterile plants through tissue-specific expression of CKX1 and gai genes that are involved in oxidative cytokinin degradation and gibberellin signal transduction. In this dominant male sterility system, the male-sterile phenotype is achieved in transgenic plants that are homozygous for the transgene, and it is reversible by exogenous hormone applications.

Alternatively, fertility can be induced through environmental conditions. In rice TGMS (thermosensitive genetic male sterility) and PGMS (photoperiod-sensitive

genetic male sterility) mutants, male sterility is influenced by temperature and photoperiod length (He 1999; Dong et al. 2002). The temperature occurring just after panicle initiation is the most critical in the expression of fertility and sterility. Most rice TGMS lines are male fertile at temperatures under 25 °C and sterile at higher temperatures (Sun et al. 1989). The seeds of TGMS lines are multiplied by selfing when exposed to the right temperature at the critical growth stage. PGMS lines are fertile under natural short-day and male sterile under long-day conditions. In this system, the male-sterile female line can be propagated by growing it under environmental conditions that restore fertility. This approach requires no restorer lines and no chemical treatment. However, controlled environmental conditions are needed to avoid the plants to be constantly challenged by unfavorable fluctuations in their environment. Other conditional male fertility systems are based on a repressor of the male sterility gene or on the inducible expression of a fertility restorer gene that complements the defect (Cigan and Albertsen 2000).

The breeding and large-scale adoption of hybrid seeds is an important achievement in agriculture. Rice hybrid seed production uses cytoplasmic male sterile lines or photoperiod-/thermosensitive genic male sterile lines (PTGMS) as female parent. Cytoplasmic male sterile lines are propagated via cross-pollination by corresponding maintainer lines, whereas PTGMS lines are propagated via self-pollination under environmental conditions restoring male fertility. Despite huge successes, both systems have their intrinsic drawbacks. Here they constructed a rice male sterility system using a nuclear gene named *Oryza sativa* No Pollen 1 (OsNP1). OsNP1 encodes a putative glucose–methanol–choline oxidoreductase regulating tapetum degeneration and pollen exine formation; it is specifically expressed in the tapetum and miscrospores (Chang et al. 2016).

Male sterility was reported first time by Achimowitsh in 1931 on sugar beet. Genes governing photoperiod sensitive male sterility in rice was characterized by Mei et al. (1999) and Zhang et al. (1994). In these studies, pms1, pms2, and pms3 genes were localized on chromosome 7, 3, and 12. The effect of pms1 on pollen fertility was much larger than that of pms2 (Zhang et al. 1994). Adenine phosphoribosyltransferase (APRT) is the major enzyme for converting adenine intoadenosine-3′-phosphate (AMP) associated with the purine salvage pathway. APRT gene mutation causes the male sterility in *Arabidopsis* (Gaillard et al. 1998). In rice, APRT gene and TGMS phenotype were investigated (Li et al. 2003). The findings indicated that the APRT gene contains six introns. The transcription of APRT gene is downregulated in TGMS rice line Annong S-1 by high temperature stress (28 °C) that perhaps results in the pollen abnormal development (Li et al. 2003).

10 Engineering of Male Sterile Mutants

One way to achieve male sterility is the use of gene that encodes a protein which is able to disrupt cell function (Mariani et al. 1990, 1992; Burgesset al. 2002). Barnase/Barstar is a useful system for male sterility using tapetal-specific promoter TA29 that

drives expression of the barnase gene, inducing male sterile plants (Mariani et al. 1990, 1992). In transgenic *Nicotiana tabacum* plants, the argE gene products that were controlled by tapetal-specific promoter Ta29 lead to empty anthers, resulting in male sterile plants (Kriete et al. 1996). Similarly, Roque et al. (2007) fused the PsEND1 promoter to the barnase gene which permits identification of male sterile line before flowering. The PsEND1 is an anther-specific promoter that drives gene expression in a tightly specific pattern restricted to developing anther. This promoter was widely applied in species such as *Arabidopsis*, oilseed rape, rice, and wheat (Gomez et al. 2004; Pistón et al. 2008). The other way to introduce male sterility is the use of diphtheria toxin A-chain that is expressed in a tissue-specific manner (Koltunow et al. 1990). A novel gene MSP1 (multiple sporocyte) that controls early sporogenic development was elucidated by Nonomura et al. (2003). Results implicated that the MSP1gene encodes a leu-rich repeat receptor-like protein kinase. The formation of anther wall layer was disordered and the tapetum layer was lost completely because of the expression of the msp1 gene mutation.

11 Category of Rice CMS

Breeders of China have developed more than 60 types of series of elite CMS lines. Based on the origins of the cytoplasms including the WA-type, Dian1-type, Honglian-type, Gambiaka-type, K-type, and Maxie-type pollens, like the CMS-HL type, no sterile plants have been observed in the F2 population. In a few CMS lines derived from crossing between wild rice within the AA genome species and rice cultivars as different parents have different restoring characteristics to known CMS types. For example, two CMS lines, IR66707A and IR69700A, developed by IRRI have very stable male sterility. But, their cytoplasms come from *O. perennis* and *O. glumaepatula*, respectively, that shares the nuclear genome of cultivated rice IR64, a strong restorer for WA-, HL-, and CMS-BT (Dalmacio et al. 1995).

12 CMS-WA

As mentioned CMS-WA is one of the types of CMS in this group. It includes the indica CMS lines currently commercialized in hybrid production such as Aibai, Tenye, Indonesia rice-type, K-type, Gambiaka-type, Dissi-type, Maxie CMS, etc. The CMS lines in this group are developed from crosses between traditional rice varieties (*O. nivara*, *O. rufipogon*, *O. glaberrima*, and *O. sativa* ssp. *indica*) as a maternal parent with early-matured indica rice varieties (such as Zhen-Shan 97, Xieqingzao, Gu Y-12, II-32, etc.) as recurrent paternal lines. Pollen abortion occurs relatively earlier during microspore development, at the uninucleate stage mainly. Most of the pollens are irregularly shaped and unstainable with I2-KI solution. More than 90% of pollens are normal in F1 hybrids and the sterile, and the fertile pollens are segregated in F2 generations as 3:1 or 15:1 (fertile to sterile) indicating one or two restorer alleles in different combinations. The male sterility of

these CMS lines is very stable under various environments, and the restoring possibility of this group is confined within the AA genome species. The sterility of this CMS type is usually maintained by the early-maturing semi-dwarf indica varieties originating in China.

13 CMS-HL

By backcrossing a red-awned wild rice in Hainan Island (*O. rufipogon*) as the maternal parent with the recurrent paternal parent Lian-TangZao, an early-matured indica variety by Wuhan University in 1974, the original Honglian CMS line, is developed. The pollen abortion usually occurs at the dinucleate stage; these sterile pollens are spherical and negatively stainable with I2-KI solution. Although about 50% of normal pollen in F1 hybrids is derived from Honglian CMS lines, their seed setting is normal. No fertility segregation is observed in the population of F2 generations, showing typical gametophyte features. The relationship between restorers and maintainers of Honglian CMS lines tends to be contrary to that of CMS-WA lines. The restoration spectrum of the Honglian type is broader than that of the WA CMS within AA genome species.

14 BT-CMS

Apart from the CMS-WA and HL, another type of CMS is CMSBT. This CMS group also includes Dian-1 and Dian-3 CMS lines.

They are all developed by nuclear substitution via backcrossing of the conventional indica varieties (E-Shan-Ta-Bai-Gu, ChinsurahBoro II) as the maternal parent with Chinese japonica varieties such as Taichung 65 as the recurrent paternal parent. The pollens in this CMS group usually abort at the trinucleate stage. The abortive pollens are spherical and stainable with I2-KI solution. Normally, the pollens show complete sterility, and anthers do not dehisce unless under conditions of high temperature and low humidity. However, they share a similar restoration and maintenance relationship with the CMSHL type (Hu and Li 1985).

They have a relatively broader restoring spectrum than that of CMS-WA. The F1 hybrids derived from them have a normal seed setting with about 50% fertile pollens, like the CMS-HL type, and no sterile plants have been observed in the F2 population. In addition, a few CMS lines derived from outcrossing between wild rice within the AA genome species and rice cultivars as recurrent parents have different restoring characteristics to known CMS types. For example, two CMS lines, IR66707A and IR69700A, developed by IRRI have very stable male sterility. However, their cytoplasms come from *O. perennis* and *O. glumaepatula*, respectively, sharing the nuclear genome of cultivated rice IR64, a strong restorer for WA-, HL-, and CMS-BT (Dalmacio et al. 1995). Their male sterility could not be restored by any of the known restorers also reported the development of three CMS lines of M01A, M02A, and M03A from a cross between Dongxiang wild rice (*O. rufipogon*

Griff) and MM872 (*O. sativa* L. ssp. *indica*). The three CMS lines showed 100% male sterility with partial anther defectiveness, and the male sterility could be restored by some of the rice lines derived from Dongxiang wild rice only, rather than Minghui63, a strong restorer line for WA-, HL-, and CMS-BT. Which means these CMS lines should belong to a new CMS group. It suggests that many cytoplasms with different CMS loci exist in the AA genome species and they are prone to the discipline of "gene-to-gene" for the restoration of fertility.

With rapid development of molecular genetics and genomics of rice, molecular mapping has been a very effective tool to precisely estimate the genetic distance, gene location, and the effect of the fertility restoration of rice for hybrid rice breeders. Currently, eight chromosomal loci for Rf genes have been proposed in earlier published studies, one on chromosome 1, two on chromosomes 7 and 10, four QTLs on chromosomes 2, 3, 4, and 5, and two major QTLs on chromosome 10 (Tan et al. 1998).

With the knowledge on hybrid rice, its mode of development and sources like CMS in their production there has been a huge development in availability of this staple food, rice. An in-depth research on combination of recombinant DNA technology and genetic transformation method opens new avenues for induction of male sterility which will be extremely valuable for hybrid seed production. Yet another challenge is to identify and use pollen-specific promoters in the process of developing MS lines of rice which can be further used in the breeding programs.

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Male Sterility System for Hybrid Rice Breeding and Seed Production

Nimisha Amist and N. B. Singh

Abstract

Heterosis has been exploited for production of commercial hybrid seed, and rice is the most significant model crop plant where seed production has occurred through multiple types of male sterile lines. Rice being a staple crop throughout the world, cultivation of rice hybrid varieties is escalating. Isolation and identification of male sterility-related genes and proteins will help in acquiring information about the reasons and mechanism behind occurrence of male sterility and key players involved in microspore abortion. Recently, through the experimental studies involving genetics or proteomics, genes and proteins associated with cytoplasmic male sterility (CMS), photoperiod-sensitive male sterility (PSMS), self-incompatibility and microspore deterioration have been identified. In plants, mitochondrial genes along with nuclear genes or nuclear genes alone can induce male sterility, and consequential conditions known as CMS and genetic male sterility (GMS) occur, respectively. CMS and GMS assist in hybrid seed production and as a result permit breeders to exploit yield gains connected with hybrid vigour. The communication between mitochondrial and nuclear genes regulates male specificity, occurrence and restoration of fertility under CMS system. Genes of nuclear restorer of fertility (*Rf*) helps in suppressing CMS. Thus, CMS/*Rf* systems present a typical model for evaluating mitochondria-nuclear genes interactions in plants. CMS/*Rf* scheme proved to be cost-effective and efficient tools for the production of hybrid seeds. However, environment-sensitive GMS (EGMS) mutants engage epigenetic control through noncoding RNAs and are capable of regressing fertility beneath diverse growth conditions, making them valuable breeding materials in the hybrid seed industry. In this chapter, our focus is on the phenomenon of male sterility along with recent progress in studies of the three major CMS/*Rf* systems in rice.

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Keywords

Anther · Cytoplasmic male sterility · Genetic male sterility · Heterosis · Hybrids · Microsporogenesis · Rice

1 Introduction

Male sterility is treated as an exceptional gift to mankind from nature. Male sterility has contributed massively in fighting global hunger by helping in development of high-yielding hybrids of various food crops. In male sterility, the male reproductive parts of a plant do not participate in natural process of sexual reproduction as they are non-functional or aborted after formation or are absent. The defect in development during any step of microsporogenesis, i.e. from spore mother cell division to the release of pollen grains, leads to abnormality in the male reproductive system. The plants with damaged anther in natural conditions were first reported by Kolreuter (1763). It was Darwin (1877) who acknowledged the significance of this phenomenon and theorized that the loss of reproductive capability of plant may help in evolution by improving adaptation in the course of gene transfer from a variety of related and unrelated individuals through cross-pollination. However, male sterility can be utilized in plant breeding only if individuals with distorted male fertility maintain their female fertility. Viable seeds are produced from male sterile plants when fertilized with pollen grains from other plants which are dispersed through external agencies like wind, insect, etc. In the past, a mutant with male-sterility had integrated in the populations of cultivars and germplasm naturally, but as their economic value was not estimated at that time, they were easily lost over a time of few generations. However, with the progression in perception of heterosis (Shull 1908), the prospective benefits of male sterility through which the productivity can be enhanced were realized. The production of hybrid seed using male sterility was first reported in sorghum (Stephens 1937). Jones and Emsweller (1937) confirmed use of male sterility in production of hybrid seed in onion. The male-sterile trait can be produced in nature either through mutations or by induction of mutagenesis or hybridization and selection.

Male sterility garnered a greater significance in relation to detailed economic studies performed to gather information about the genetics, physiology and genomics behind the phenomenon. In the present chapter, we have attempted to summarize available literature on various aspects of male sterility, its origin and utilization in major crop rice.

2 Different Male Sterility Systems

Male sterility is an anomaly that is rarely observed in higher plants. In this condition, individual's male reproductive system fails to contribute in creation of its progenies. Male sterility can arise due to various reasons like failure of anther tissues to mature and differentiate normally, malfunction during microsporogenesis, inability of

anther in releasing the mature pollen grains and also incompatibility of mature pollen grains to germinate on the stigma surface. However the abnormalities in the male reproductive systems do not impact the female reproductive system, and fertile seeds are produced when they are cross-pollinated manually or by natural means. In view with the fact that male sterility is a display of anomalous growth and development, the expression of the genes controlling male sterility may also be varied and contradictory across the crops and genotypes. Male sterility systems have been classified into three category on the basis of irregularities of the androecium such as structural (absence or deformity of anthers), sporogenous (defective microsporogenesis) and functional (failure of mature pollen to germinate). However, it has also been classified into genetic, cytoplasmic and cytoplasmic nuclear (or genetic) male sterility based upon genetic control mechanisms.

2.1 Genetic Male Sterility

Genetic male sterility (GMS) is the most frequent form of male sterility found in a number of plant species in both monocots and dicots (Kaul 1988). In this system it is the nuclear genes which control the male sterility, and it is totally independent of cytoplasm. Independently segregated, one or two pair of recessive genes generally controls the expression of genetic male sterility. However, a few exceptions are also found where one or two dominant genes controlled the male sterility. Morphological characters like stem pigmentation, translucent anthers, sparse podding, delayed flowering, etc. have been associated with the male sterility (Kaul 1988; Verulkar and Singh 1997). Unexpectedly mutant male-sterile plants are introduced in the population, and usually they carry homozygous alleles (*msms*). This male sterility will be lost if not maintained as heterozygotes (*Msms*). In order to maintain genetic male sterility, the mutants with male-sterility should be pollinated with fertile homozygous (*MsMs*) or a heterozygote (*Msms*) (Saxena and Hingane 2015). However, it is difficult to maintain male sterility through reproductive methods if the dominant allele is controlling the sterility.

2.2 Cytoplasmic Male Sterility

The genes in cytoplasm govern this type of male sterility. The gene regulating the cytoplasm is defective mitochondrial DNA. The detrimental interactions between the mitochondrial and nuclear genes are responsible for induction of cytoplasmic male sterility (CMS). This type of cytoplasm is considered as "sterile" (S), and it can arise impulsively in nature or in the course of hybridization. Such plants create non-fertile pollen grains as its nucleus also possesses a pair of recessive non-restoring (*msms*) alleles. The maintenance of cytoplasmic male sterility depends upon the genotypes in which non-restoring recessive nuclear alleles are present and it carries fertile (F) or normal (N) cytoplasm (Fig. 1). Nearly 150 plant species have been reported to exhibit this male sterility (Kaul 1988). In the cytoplasmic male

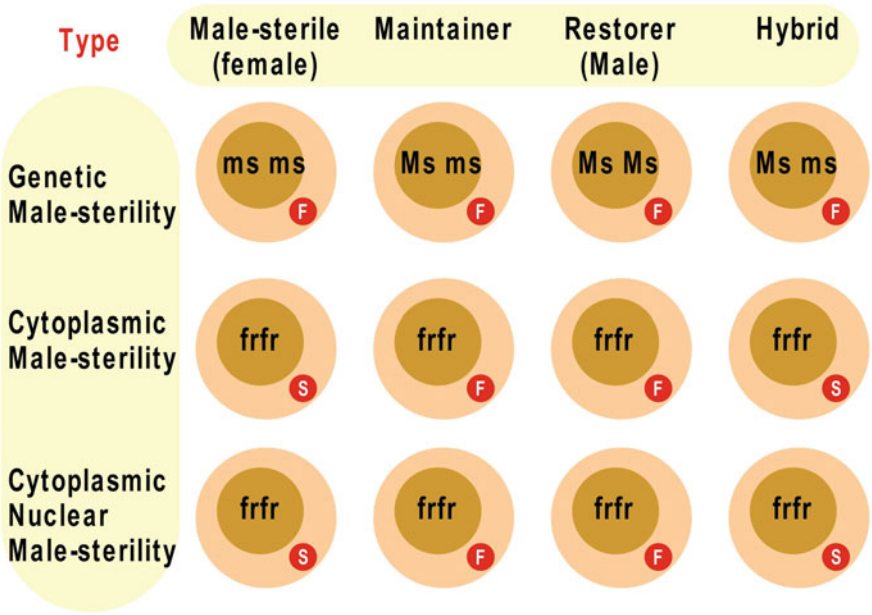

Fig. 1 Diagrammatic representation of genetic structure of the nucleus and cytoplasm of the three male sterility systems (modified after Saxena and Hingane 2015)

sterility system, integration of recessive non-restorer nuclear alleles prevents the deviation of hybrid parents. Normally, male hybrid parent should resemble its maintainer at genotypic level, but its nuclear genome should be varied and should have capability of producing heterotic hybrid progenies. The biggest drawback of cytoplasmic male sterility is the deficiency of fertility restoring genes due to which it cannot be used for field crops, and there are complications in generation of large amount of hybrid seed. Instead, this system has been used in horticultural crops where fruits are consumed or the seeds are of no commercial value.

2.3 Cytoplasmic Nuclear Male Sterility

Cytoplasmic nuclear male sterility (CNMS) is just like cytoplasmic male sterility as in it also the expression of male sterility is an outcome of communication between cytoplasmic and nuclear genomes. The variation between the two types is through fertility restoration mechanisms. In the CMS the fertility is controlled by normal cytoplasm of the maintainer lines, while in CNMS dominant fertility-restoring genes are found in the nucleus of restorer line. Hence, it is referred as cytoplasmic nuclear/genetic male sterility. Furthermore, the expression of male fertility or sterility could be total or partial which is controlled by the type of fertility-restoring gene. Occasionally existing environmental conditions like photoperiod, temperature, or both

also regulate the expressions of the gene. However, this type of male sterility has been widely used in hybrid breeding programs in a number of field crops. There exist three distinct types of genotypes in this complete hybrid system:

- First type (I) of line is the male-sterile female line with sterile cytoplasm and recessive fertility nuclear alleles (*frfr*).
- Second line (II) is for maintenance of the female line, and it possesses fertile cytoplasm and recessive nuclear alleles (*frfr*). Entire progeny of male sterile is produced through a cross between first and second type of lines.
- Third parent (III) is nominated as restorer line, and it includes dominant fertility-restoring gene (*FrFr*). The cross between first type and this third type of line results in production of hybrid plant with restored male fertility.

Molecular studies have revealed that the reorganization of mitochondrial genome that are related with synthesis of toxic proteins and decline in respiration regulated the expression of this male sterility system. Although various hypotheses have been proposed, molecular basis of this male sterility is not well recognized among crops. Chimeric mitochondrial ORFs (open reading frames) have discovered to be linked with male sterility. In rice the expression of male sterility has been coupled with ORF which encodes a cytotoxin peptide (Wang et al. 2006). It was proved experimentally that anomalous copy of a mitochondrial gene created abnormal mRNA transcripts which included an additional ORF (Iwabuchi et al. 1993). Modification in promoter regions and in segment of coding regions of mitochondrial ATP synthase might be related with male sterility (Hanson and Bentolila 2004). The variation in coding segment might possibly damage the action of ATP synthase. The male sterility can be activated in the pigeon pea through 13 ORFs discovered in the mitochondrial genome during genomic study (Tuteja et al. 2013).

2.4 Environment-Sensitive Genic Male Sterility

Environment-sensitive genic male sterility (EGMS) is an elite type of male sterility system. The environmental factors direct the expression of male sterility and fertility of the plants. In this system, environmental conditions like low or high temperature, short or long photoperiod, variable light intensity, different soilborne stresses or their specific combinations regulate expression of the male sterility gene (Kaul 1988). EGMS can happen mutually in genetic as well as cytoplasmic nuclear male sterility systems. The reversion of male sterility to male fertility is influenced by cytoplasmic rather than nuclear genetic factors (Levings et al. 1980). The EGMS line was earliest reported in rice by Shi (1981), and later it was used in hybrid breeding program. In EGMS the second type of maintainer line is not required; thus this hybrid system is universally called as “two-parent hybrid” breeding. The alteration of male sterility to fertility and its reversal is an intricate genetic occurrence, and additional research is necessary at genomics and physiological levels to identify it better.

3 Role of Cytoplasmic and Genetic Male Sterility in Hybrid Seed Production Through Heterosis

Hybrid vigour or heterosis is an incident in which the offspring obtained from a cross of two inbred lines surpass the parent lines. It has been observed that yield of hybrid crops was about 15–50% higher than inbred varieties (Tester and Langridge 2010). Thus exploitation of heterosis has created incredible economic benefits in the field of crop production worldwide. In fact, production of more than half of the major crops like maize, rice, sorghum, rapeseed and sunflower occurs through hybrid varieties (Li et al. 2007). Production of hybrid seeds in self-pollinating plants necessitates removal of anthers or functional pollen grains to avoid self-pollination. However, during middle of twentieth century, hybrid seed production was an expensive process as emasculation was done with help of manual labour, machines or chemical treatments and it was even detrimental to the environment. The biggest advantage of CMS and EGMS lines is that both lines doesn't require emasculation and are consequently perfect female lines for hybrid seed production. In the 1950s, hybrid corn was produced for the first time using the maize CMS-T (Texas) system. This system enhanced the competence of hybrid seed production and improved yields of maize. Soon after, CMS-based hybrid seed production technology was developed for many crops, including rice. In year 1976, first commercial hybrid rice was released in China in which the grain yield increased by over 20%, and since the late 1980s it has accounted for approximately 55% of the total rice planting area in China (Cheng et al. 2007).

Hybrid seed production technology uses two systems, i.e. three-line and two-line system. Three-line system is used in CMS-based hybrid seed technology, and this system requires three diverse breeding lines, i.e. the CMS line (female parent), the maintainer and the restorer lines (Fig. 2a). The female parent used has a male sterile cytoplasm in which CMS-causing gene is present, and it has a non-functional nuclear restorer gene or genes (*Rf*, or restorer) for fertility (Schnable and Wise 1998). The maintainer line serving as the male parent in cross for the breeding of the CMS line possesses normal fertile cytoplasm, but the nuclear genome is the same as the CMS line. The restorer line has an operative *Rf* gene or genes and therefore serves as the male parent in cross between the CMS line and restorer for creation of F1 hybrid seeds. The *Rf* gene reinstates male fertility in the F1 plants, and the amalgamation of nuclear genomes belonging to CMS and the restorer line generate hybrid vigour.

In contrast to CMS, GMS mutants are not apt for hybrid seed production as their male-sterility qualities are not preserved efficiently. GMS is regulated by a pair of recessive genes, and it requires heterozygote forms for maintenance. The procedure of the seed production in GSM is not simple and needs extra precaution. The heterozygote (*Msms*) seeds are sown in secluded area where they segregate following Mendelian principles and generate about 50% fertile and 50% sterile plants. It is important to tag two types of plants. However, after maturity the seed from the male-sterile plants should be harvested, which have been pollinated by heterozygote fertile segregates. The seed from male and female parents should be sown in isolation, and

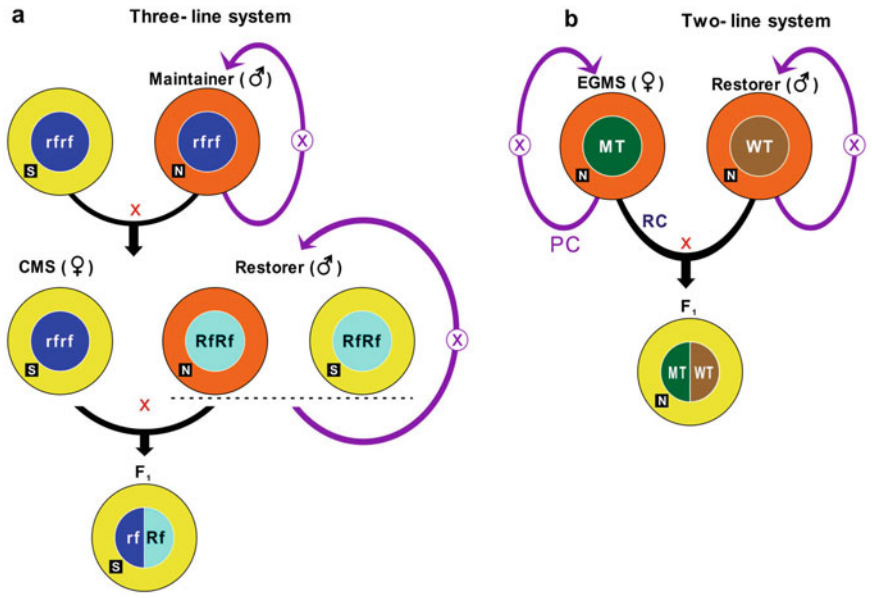

Fig. 2 Hybrid seed production through a three-line system and two-line systems. (**a**) In three-line system CMS line contains sterile (S) cytoplasm and a non-functional (recessive) restorer (rf) gene or genes; a maintainer line contains normal (N) cytoplasm and a nuclear genome identical to that of the CMS line; restorer line: normal (N) or sterile (S) cytoplasm and an efficient (dominant) restorer (Rf) gene or genes. (**b**) In the two-line system, an EGMS mutant (MT) line is propagated by self-pollination when grown under tolerant conditions (PC); RC, restrictive conditions for reverse PGMS, or high-temperature conditions thus serves as the female parent for crossing with a wild-type (WT) line to produce hybrid seeds (adapted and modified after Chen and Liu 2014)

the female rows will separate out for fertility/sterility. It is very important to remove fertile segregates before they start releasing pollen for maintenance of quality of the hybrid seed. This will allow crossing of the male-sterile plants with selected male parent only. However, if the male sterility gene is correlated to any morphological characteristic, then identifying and separating becomes uncomplicated. The production of hybrid seed with GSM lines is very complicated and entails superior attention and resources.

EGMS mutants have facilitated in the utilization of some of the GMS traits in hybrid crop breeding (Virmani and Ilyas-Ahmed 2001). The environmental factors like day length and temperature determine and alter pollen fertility of EGMS lines. In the year 1973 Nongken 58S (NK58S), the first photoperiod-sensitive GMS (PGMS) mutant was discovered in japonica rice (*Oryza sativa* ssp. japonica). NK58S male fertility is determined by the day length, and a male sterility is observed when developed under long-day conditions, but male fertility is maintained through short-day conditions (Shi 1985). Annong S-1, a temperature-sensitive GMS (TGMS) mutant, was also reported in indica rice (*O. sativa* ssp. indica) in 1988. High temperature induces male sterility in Annong S-1, while low temperature

results into male fertility (Deng et al. 1999). The restoration of male fertility in PGMS and TGMS facilitate hybrid seed production through a two-line system. A PGMS or TGMS line grown under controlled conditions (long-day or high-temperature conditions) functions as the male-sterile female parent. This same line can be propagated under tolerant conditions (short-day or low-temperature conditions) (Fig. 2b). The biggest advantage of two-line system is that it eradicates the necessity for crossing in order to propagate the male-sterility line. Furthermore, all regular varieties have the alleles of wild-type fertility gene that can reinstate male fertility, and therefore they might be used as the male parents for hybridization. Consequently, two-line systems abridge hybrid seed production mechanism and decrease costs. Approximately, about 20% of the total hybrid rice planting area in China is occupied by two-line hybrid rice based on PGMS or TGMS (Li et al. 2007). The seed production system involving two-line hybrid is fascinating, and selection of production sites is the most significant. It is important to use two sites with distinct temperature and photoperiod in two-line system. These sites must in addition satisfy the requisite length of photoperiod. The first site should have the temperature range which helps in maintenance of male sterility line, and as a result all plants are male sterile in the first site and are used for hybrid seed production. The male and female lines are grown in definite ratio and are cross-pollinated. The seeds are collected from the male-sterile rows. In support of the continuation of male-sterile line, its seed is grown at second site. At this site suitable temperature is maintained which can induce male fertility, and all the plants produced are male fertile. Therefore, the seed produced from second site will be the self-pollinated. The next season can be used for the second cycle of seed production.

4 Current Progress on Cytoplasmic Male Sterility and Restorer Genes in Rice

A number of wild plants have CMS cytoplasm but are male fertile due to the presence of *Rf* genes in their nuclear genomes. Thus, the discovery of CMS cytoplasms is usually through genetic interbreeding or somatic hybridization (protoplast fusion) that segregates the CMS cytoplasm from its nuclear *Rf* gene(s). Cross among the probable CMS lines as female parents and non-restorer line (i.e. lines having recessive rf alleles) of the same or different species can be followed by selection of male-sterile progeny. The genes of CMS contender can be recognized by numerous strategies. The most common technique is to investigate for variation in the mitochondrial genome along with discrepancy in the transcriptome of mitochondria or proteins of CMS cytoplasm lines with and without the *Rf* gene(s). Around 43 probe sequences defining the whole mitochondrial genome of rice were utilized to perform RNA-blot analysis for cloning the gene for rice CMS-WA line (Liu et al. 2007). CMS-WA is a male-abortive wild rice (*Oryza rufipogon*)-dependant CMS system that is most extensively utilized for hybrid rice breeding consisting of the CMS-WA line, maintainer line and fertility-restored lines with the restorer gene *Rf3* or *Rf4*. In the end of analysis, a transcript precise to the

CMS-WA line was detected, and its large quantity was decreased in the *Rf4*-restored lines.

The reorganization of mitochondrial genome results into formation of several CMS genes. Table 1 summarizes seven types of CMS from rice species. About ten genes have been found to be involved in synthesis of CMS genes, and they are crucial mitochondrial genes belonging to the mitochondrial electron transfer chain pathways. It appears that genes *cox1*, *atp8* and *atp6* are recurrently implicated in the derivation of CMS genes. Almost all the CMS genes encode transmembrane proteins (Table 1). In rice, small proteins with an N terminus parallel to COX1 and the left over SUO (sequence of unknown origin) portion are encoded by *orf79* genes in CMS-BT and through its variant *orfH79* gene in CMS-HL (Wang et al. 2006) (Fig. 3).

In rice, there are three main CMS/RF systems, which are named as CMS-BT, CMS-WA and CMS-HL. They have been defined by distinct genetic and cytological features (Table 1, Fig. 3).

4.1 CMS-BT

The BT-type rice is the most accurately characterized system of CMS in rice. In CMS-BT type, the male sterility is caused by the cytoplasm obtained from the Chinsurah Boro II rice line which is then crossed with the nucleus of Taichung65 rice line that is lacking restorer gene (Shinjyo 1969). A chimeric gene called *orf79* present downstream to *atp6* is reported from the mitochondrial genome of Chinsurah Boro II, and this gene encodes for cytotoxic peptide and has been established as a responsible gene for the gametophytic male sterility of CMS-BT rice through transgenic experiments (Iwabuchi et al. 1993; Akagi et al. 1994; Wang et al. 2006). A nuclear gene, *Rf1*, which encodes a pentatricopeptide repeat-containing protein controls the fertility restoration of CMS-BT rice (Kazama and Toriyama 2003; Akagi et al. 2004; Komori et al. 2004). The locus of *Rf1* is reported to consist of two *Rf* genes *Rf1a* and *Rf1b* (Wang et al. 2006). The two *Rf1* genes help in restoring fertility of CMS-BT rice by repressing the *orf79* transcript, but the mechanism adapted by both is different. RF1A silences transcript by endo-nucleolytic cleavage, while RF1B causes degradation of the di-cistronic RNA. On the other hand, if both *Rf1* genes are present, then *Rf1a* gene imposes epistatic effect over the *Rf1b* gene in their RNA processing (Wang et al. 2006). It appears that partially degradation of processed co-transcript *B-atp6-orf79* occurs, but the unprocessed *B-atp6-orf79* RNA still has the ability to translate. Thus, as a result a definite quantity of ORFH79 protein accumulates in transgenic rice, yet a fertile phenotype was exhibited in the transgenic line (Kazama et al. 2008). The obtained results reflect that a definite level of accretion of ORF79 in rice do not induce CMS. There is a need to investigate the obligatory amount of ORF79 protein required for the pollen abortion.

Table 1 Identified cytoplasmic male sterility (CMS)/restorer (*Rf*) gene systems in rice

S. no	CMS type	CMS gene	*Rf* locus	Mechanism of *Rf*	CMS gene encoded proteins property	CMS gene encoded proteins property	Reference(s)
1.	CMS-BT (gametophytic)	B-*atp6-orf79*	*Rf1a*, *Rf1b*	RNA post-transcriptional level	Membrane protein	Pentatricopeptide repeat protein	Akagi et al. (2004), Kazama and Toriyama (2003), Komori et al. (2004) and Wang et al. (2006)
2.	CMS-HL (Gametophytic)	*atp6-orfH79*	*Rf5* (*Rf1a*)	RNA post-transcriptional level	Membrane protein	Pentatricopeptide repeat protein	Hu et al. (2012) and Wang et al. (2013)
3.	CMS-WA (sporophytic)	*rpl5-WA 352*	*Rf3*, *Rf4*	Protein translational or post-translational, RNA post-transcriptional level	Membrane protein	Unknown	Luo et al. (2013), Zhang et al. (1997, 2002)
4.	CMS-LD (gametophytic)	L-*atp6-orf79*e	*Rf2*	Protein translational or post-translational	Unknown	Glycine-rich protein	Itabashi et al. (2009, 2011)
5.	CMS-CW (gametophytic)	*orf307*	*Rf17*	Protein translational or post-translational	Unknown	Acyl-carrier protein synthase	Fujii et al. (2007), Fujii and Toriyama (2009) and Fujii et al. (2010)
6.	CMS-RT120	*rpl5-orf352*	*Rf102*	Unknown	Membrane protein	Unknown	Okazaki et al. (2013)
7.	CMS-RT98	*orf113-atp4-cox3*	Unknown	Unknown	Membrane protein	Unknown	Igarashi et al. (2013)

Source: Adapted and modified after Chen and Liu (2014)

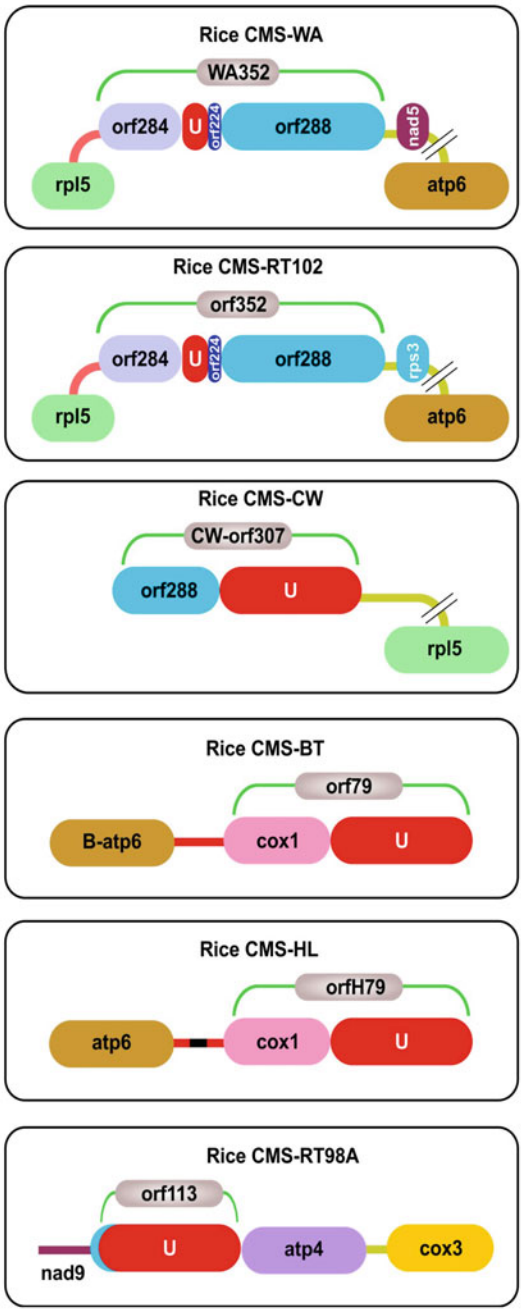

Fig. 3 Diagrammatic representation of structure of cytoplasmic male sterility (CMS)-related genes of rice species. The oval boxes stand for coding sequences, and the straight lines designate flanking regions of the open reading frames (ORFs). The same colours indicate the similarity between the sequences of the same genes (including flanking and coding sequences). Red boxes symbolize sequences of unknown origin (U). Diagrams do not represent dimension existing natural system (adapted and modified after Chen and Liu 2014)

4.2 CMS-HL

The red-awned wild rice (*Oryza rufipogon*) from Hainan Island, China, was repeatedly backed crossed with almost mature indica variety called Lian-Tang-Zao resulting in the formation of CMS-HL line of rice. CMS-HL-based hybrid rice varieties have been extensively grown in China due to advanced agronomic properties and production of superior quality grain than those varieties belonging to other CMS systems (Liu et al. 2004). CMS-HL has been investigated more efficiently in comparison to CMS-BT and CMS-WA. The increase in accretion of reactive oxygen species (ROS), drastically declined adenylate content along with ATP/ADP ratio have been observed and even the membrane potential of mitochondrial membranes also decreased in Yuetai A when compared with Yuetai B during microsporogenesis (Li et al. 2004; Wan et al. 2007). In CMS system of *Brassica napus* and *Nicotiana tabacum*, the same incident of variation in membrane potential and ATP/ADP ratio was also reported (Bergman et al. 2000; Teixeira et al. 2005). The research findings suggested that mitochondrial activity was severely required for gametophyte development as lower ATP levels were also recorded during vegetative growth, but only function of pollen grain was lost. The mitochondrial chimeric gene called *orfH79* situated downstream of *atp6* has been recommended as the gene causing the CMS trait of Honglian rice (Yi et al. 2002). In a transgenic experiment, it was proofed that expression of *orfH79* caused abortion of pollen in HL-maintainer line (Peng et al. 2010). The restoration of fertility is through nuclear *Rf5* gene in CMS-HL rice. Moreover, *Rf5* directly interacts with a Gly-rich protein, GRP162 resulting into a formation of subunit for re-establishment of fertility complex. The CMS allied transcript *atp6-orfH79* processed by fertility complex instigates a new outlook on the molecular mechanism underlying fertility reinstatement (Hu et al. 2012). The analysis of protein expression profile of Yuetai A and Yuetai B helped in understanding the molecular mechanism of CMS-HL rice. The level of proteins related with energy production reduced in the anther of CMS-HL rice (Wen et al. 2007; Sun et al. 2009) signifying that a low intensity of energy production plays a significant role in induction of CMS-HL. It appears that accretion of ORFH79 in mitochondria damages the regular functions, it decreases the level of proteins involved in energy production in anther and the mitochondrial activity is also downregulated in CMS-HL rice. The observed results reflect that defect in mitochondrial complex induces the sterile line. However, even after detailed study of CMS allied mitochondrial genes in rice, the exact mechanism behind abortion of pollen in CMS line is not clear. There is a need for development of more handy and responsive systems to study the mechanism of CMS in rice and other plants.

4.3 CMS-WA

Rice CMS-wild abortive (CMS-WA) system is obtained from the common wild species *Oryza rufipogon* Griff, which is one of the most often used for production of hybrid rice (Lin and Yuan 1980). The genetic and cytological properties of CMS-WA vary from that of CMS-BT and CMS-HL. The pollen aborts at an early

stage of microspore development in CMS-WA primarily at the uninucleate stage. The male sterility is sporophytic type and the aborted pollens are shapeless. The male sterility is gametophytic type in both CMS-BT and CMS-HL rice. The CMS-WA molecular mechanism is not well understood in comparison to that of CMS-HL and CMS-BT. Earlier, a transcript of *orfB* gene which is an unedited 1.1 kb mitochondrial gene was considered as a contender for CMS-WA sterility gene (Das et al. 2010). However, recently, *orf126* has been suggested as a contender for CMS-related gene based on evaluation of mitochondrial genomes through next-gen sequencing (Bentolila and Stefanov 2012). The genes of CMS and *Rf* in CMS-WA haves been recognized and cloned.

4.4 EGMS in Rice

The summary of EGMS genes is presented in Table 2. The first spontaneous photoperiod-sensitive genic male-sterile (PGMS) mutant discovered is Nongken 58S (NK58S) first found in the japonica cultivar Nonken58 (NK58). The discovery of NK58S stimulated large-scale utilization of two-line hybrid rice in agriculture (Shi 1985; Shi and Deng 1986). The day length regulates the fertility of NK58S at specific inflorescence maturity stages. The day length longer than 14 h during anther development causes male sterility, while shorter day length induces fertility. Temperature also plays role in maintaining male fertility by modulating the photoperiods. High level of temperatures promotes absolute male sterility under long-day conditions (He and Yuan 1989).

PeiAi64S (PA64S) is also a NK58S-derived line generated utilizing indica (*O. sativa* ssp. indica) for genetic background. PA64S due to its high compatibility and superior agronomic traits has developed into the most extensively used female parent for two-line hybrid rice breeding. However, the temperature regulates the fertility alteration of PA64S instead of day length. The temperature range above 23.5 °C during another development in PA64S induces male sterility, but lower temperature of 21–23 °C restores male fertility. In fact long-day (14 h) photoperiod represses the conversion of sterility to fertility under low temperatures (21–23 °C), while short-day (12 h) conditions fails to re-establish male fertility under high temperatures (Luo et al. 1992; Xu et al. 1999; Lu et al. 2007). The information about molecular mechanism behind the regulation of fertility through temperature and day length is still not clear in EGMS rice. A detailed genetic analysis revealed that a gene *pms3* located on chromosome 12 was the result of mutation which altered Nongken58 to transform into the PGMS rice NK58S. It has been revealed that *pms3* encodes beside noncoding RNA (lncRNA) named LDMAR. LDMAR is needed in an adequate amount for maintaining male fertility under long-day conditions. A single nucleotide polymorphism (SNP) is caused between NK58 and NK58S due to spontaneous G-C mutation which ultimately brings about amplified methylation in the promoter region of LDMAR, resulting into decrease level of LDMAR expression. The lowered LDMAR expression causes premature programmed cell death (PCD) during anther development under long days and consequently induces male sterility (Ding et al. 2012a). It has been reported that regulations of PGMS is

Table 2 The environment-sensitive genic male sterility (EGMS) genes in rice hybrid plants

S. no.	EGMS line	GMS type	Photoperiod (h) / temperature (°C) range	EGMS gene	Protein and function	Reference(s)
1.	NK58S	PGMS	≤13 h (F), ≥13.75 h (S)	*pms3*	Noncoding RNA	Ding et al. (2012a) and Moneger et al. (1994)
2.	PA64S	TGMS	≤23.5 °C (F), ≥27 °C (S)	*tms12-1*	Noncoding RNA/small RNA	Zhou et al. (2012)
3.	CSA	rPGMS	≥13.5 h(F), ≤12.5 (S)	*Csa*	MYB transcript regulator	Zhang et al. (2013)
4.	*Ugp1*	TGMS	≤21 °C(F), ≥28 °C (S)	*Ugp1*	UDP-glucose pyrophosphorylase	Chen et al. (2007)

Abbreviations: PGMS, photoperiod-sensitive genic male sterility; TGMS, temperature-sensitive genic male sterility; rPGMS, reverse photoperiod-sensitive genic male sterility. Letter F in parentheses indicates male fertility; a letter S in parentheses indicates male sterility. (Source: Adapted and modified after Chen and Liu (2014)

controlled through RNA-dependent DNA methylation. LDMAR promoter siRNA is connected with the DNA methylation level of LDMAR, which decreases the expression level of LDMAR and consequently induces male sterility in Nonken58S under long-day conditions (Ding et al. 2012b). PGMS in the rice line NK58S and TGMS in PA64S are expressed through gene *p/tms12-1* which encodes an exclusive noncoding RNA which generates *osa-smR5864w*, a 21-nucleotide small RNA. This product of *pms3* at the nucleotide level is similar to RNA *osa-smR5864w*, and *pms3* is accountable for the fertility of the pollen in NK58S and PA64S (Zhou et al. 2012). It appears that noncoding small RNA genes are significant regulator of male development and are modulated by cross talk between the genetic networks and the environmental conditions.

TGMS gene *Ugp1* in rice encodes a UDP-glucose pyrophosphorylase (UGPase). The deposition of callose in developing pollen is due to expression of *Ugp1*. The repression of *Ugp1* in plants causes male sterility. The cosuppression of endogenous *Ugp1* where the primary mRNA is not spliced also induces male sterility in *Ugp1* transgenic rice (Chen et al. 2007). However, the male sterility is reverted to fertility due to highly efficient splicing of the primary *Ugp1* mRNA into mature mRNA (Table 2). Thus, *Ugp1* cosuppression-induced male sterility symbolizes another type of TGMS.

R2R3-MYB transcription factor in rice known as CARBON STARVED ANTHER (CSA) regulates the expression of *OsMST8*. *OsMST8* encodes a monosaccharide transporter family member to facilitate the separation of sugar from vegetative tissues to anthers for pollen maturation (Zhang et al. 2010). The mutant of *csa* plants exhibits male sterility under short-day conditions and male fertility in long-day conditions which is a reverse PGMS trait (Zhang et al. 2013) (Table 2). The

introduction of the *csa* locus into an *indica* background from the original *japonica* mutant maintains the reverse PGMS character. The F1 hybrid progeny of cross between *indica csa* line with an *indica* fertile line demonstrates heterosis and superior yield, signifying the possible use of this reverse PGMS mutant for hybrid rice breeding (Zhang et al. 2013).

5 The Cross Talk Between CMS, EGMS and GMS

Mutants from a variety of plant species permit us to utilize genetic approach for exposing the developmental mechanisms behind the male reproductive system (Wilson and Zhang 2009; Borg and Twell 2010; Twell 2011). Important aspects have been recognized in *Arabidopsis*, and scientists have assembled a moderately inclusive pathways concerned with the development of the male reproductive system (Wilson and Zhang 2009; Twell 2011). However, the mutants of *Arabidopsis* were mainly created due to both physical and chemical mutagens like X-rays, EMS, etc., and since the genomic mutation was responsible for male sterile phenotypes, it was referred as GMS. Even though there is distinction in the cause of male sterility among the systems, CMS or EGMS and GMS have an accord in nucleus gene regulation (Fig. 4). In CMS plants, mitochondrial CMS-related proteins send retrograde signals that stimulate nuclear factors to stimulate downstream pathways that controls the fate of pollen. In case of EGMS, the signals generated by environmental

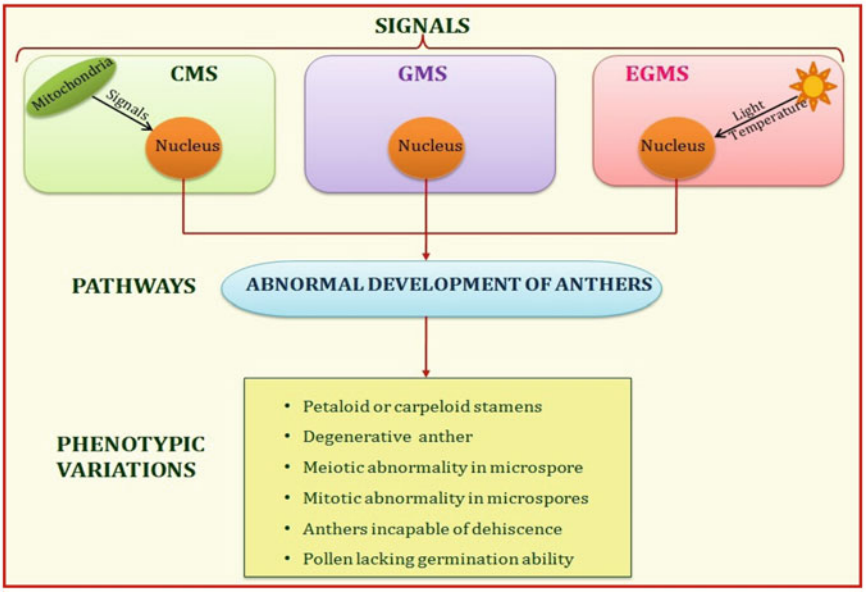

Fig. 4 The harmony in regulatory pathways of CMS, EGMS and GMS (adapted and modified after Wang et al. 2013)

factors like light or temperature are perceived by unidentified receptors and conveyed to the nucleus for directing pollen development. Consequently, it seems that the phenotype of both CMS and EGMS are created due to the signals transmitted from the nucleus. The signals must be damaging to the regular pathways that regulate the development of the male reproductive system. However in GMS mutants, the usual pathways might have been depreciated due to absence of important factors. From this standpoint, the study of GMS holds a centre place for systematically understanding the male sterility, counting both CMS and EGMS.

It has already been proven that CMS phenotypes are produced as a result of unsuited communications among the mitochondrial and nuclear genomes in different plant populations. The levels of interaction amid the mitochondrial and nuclear genes engage both the spatial and temporal regulation of CMS proteins, CMS occurrence and CMS restoration. It has been reported that nuclear mitochondrion-sorting gene (MSG)-encoded proteins target mitochondria and interrelate with the CMS proteins to control the anther-specific accumulation of most of the CMS proteins. On the other hand, only one nucleus-encoded mitochondrial protease has been recognized called as LON which has been concerned with anther-specific CMS protein accumulation (Sarria et al. 1998). The role of interaction between CMS genes and nucleus-encoded factors in the initiation CMS, like CMS restoration, was unclear for a long time (Guo and Liu 2009). Assessment of the communication between WA352 and COX11 in CMS-WA (Luo et al. 2013) and the interface amid ORFH79 and P61 in CMS-HL (Wang et al. 2013) exposed this level of mitochondrial-nuclear gene dealings. The mitochondrial-nuclear gene communications involve the key method for CMS occurrence, and it reveals the incompatibility between the cytoplasmic and nuclear genomes. The subunits of the mitochondrial electron transport chain complexes are decisive CMS protein interactors that intervene the release of retrograde signals such as ROS and cytochrome *c* to elicit abnormal PCD in tapetal cells or microspores, resulting in sporophytic or gametophytic CMS. CMS reinstatement is also another layer reflecting interaction between mitochondria and nuclear gene. RF proteins are deviated towards mitochondria and where they interact with CMS genes resulting in restoration of fertility by restraining the expression of CMS genes or eradicating the unfavourable effect of the CMS proteins by means of diverse mechanisms at the genomic DNA, RNA, protein, or metabolic level. Latest studies have exposed different retrograde regulation for occurrence and restoration of CMS, such as in the CMS-Petaloid of carrot and CMS-CW of rice systems (Fujii et al. 2007; Fujii and Toriyama 2009; Linke et al. 2003). However, small information is available on the subject of mitochondrial retrograde regulation in plants, and the issue of ROS and few unidentified mitochondrial noncoding RNAs serving as retrograde signals, as concerned by those recognized in plastids (Hotto et al. 2012), remains to be investigated.

6 Future Prospects in Research and Applications of Rice CMS Systems

One of the emerging trends in CMS research is the recognition of additional *Rf* genes and RF-interacting proteins. Among the three major CMS/*Rf* systems in rice, only the sought-after *Rf3* for CMS-WA has not been recognized (Table 1). Current investigation on a various CMS and Rf genes in rice have presented new evidences about the genetic communications among CMS and restorer genes in plants. Most *Rf* genes belonging to CMS/*Rf* systems in rice encode PPR proteins which cleavage or degradation CMS mRNAs during processing. PPR (pentatricopeptide repeat) genes belong to a big gene family in land plants, and most of PPR proteins lack RNA endonuclease activity (Barkan and Small 2014). Consequently, some apparatus with RNA-binding and/or endonuclease activity might be present in complexes, as illustrated by the CMS-HL/Rf5/Rf6 system. Thus, main focus should be on RF components and on their function in binding and degradation of CMS transcripts. In the future more emphasis should be done on removing the detrimental phenotype related with CMS systems. Panicle enclosure is one of the technical issue faced by the CMS seeds and F1 hybrid seeds in CMS lines. During panicle enclosure, it fails to expose from the flag leaves sheath, so various spikelets are not pollinated, causing decline in seed-setting rates and yield loss (Chen and Liu 2016). *Eui1* (ELONGATED UPPERMOST INTERNODE1), a gibberellic acid (GA)-deactivating enzyme, is responsible for elongation of the uppermost internode in rice (Zhu et al. 2006), and in recessive *eui1* mutant, the panicle-elongation phenotype eradicates the enclosed panicle phenotype in the CMS lines. Therefore, it offers a new genetic element in the CMS/Rf system in rice. At last, new technologies like genome-editing method will permit researchers to implicate new technologies in hybrid breeding, as well as in new types of male sterility. The breakthrough invention and application of EGMS, such as photoperiod-sensitive male sterility and thermo-sensitive male sterility (Shi 1985; Virmani and Ilyas-Ahmed 2001), have abridged two-line hybrid rice breeding system (Chen and Liu 2014). The two systems, i.e. three-line and two-line coexistence in this new age of hybrid rice production. Current studies have isolated three extensively used genes (*pms1*, *pms3* and *tms5*) that are involved in controlling photoperiod-sensitive male sterility as in NongKen58S and thermo-sensitive male sterility in AnNong S1 and also in their derivatives (Ding et al. 2012a; Zhou et al. 2012, 2014; Fan et al. 2016; Guo and Liu 2017). As a result, the selected maintainer lines of three-line hybrid rice can be efficiently converted to EGMS lines through the genome-editing technologies. For example, quick breeding of new thermo-sensitive male sterile rice lines belonging to two-line hybrid rice breeding (Zhou et al. 2016) can be induced by knocking out the gene TMS5 in influential rice varieties using CRISPR/Cas9 (Ma et al. 2015).

In the past decades, incredible advancement has been accomplished in understanding the molecular details of CMS and fertility restoration. This information will facilitate breeders to develop competent methods for broadening the genetic diversity of CMS, decreasing the probable threat of genetic vulnerability in hybrid rice production and better exploitation of heterosis in crops.

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Advancement in Tracking Down Nitrogen Use Efficiency in Rice: Molecular Breeding and Genomics Insight

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Abstract

In an effort to increase the productivity of rice, there has been a substantial increase in the application of exogenous nitrogen fertilizer but with detrimental consequences that negatively impacted the environment. Hence, it becomes extremely essential under the current scenario to reduce the application of nitrogen but devise new solutions to maximize the nitrogen use efficiency (NUE) of crops. NUE has been defined as the yield of dry matter produced per unit of nitrogen applied to the soil. Consequently, conscious efforts need to be put in to identify plants with higher yield under low N input. Identifying the QTLs as well as the candidate genes and the signaling pathways associated with several facets of NUE using molecular markers can be used as an important tool as has already been seen for other areas of research like biotic and abiotic stresses. Here in this chapter, we have talked about the use of marker-assisted technology in improving the identification of QTLs associated with NUE in rice.

Keywords

Breeding · NUE · Mapping · QTL · Yield

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1 Introduction

Worldwide, there has been an increase in population that is predicted to be about nine billion by the year 2050 (Gregory and George 2011). However, owing to the looming threats of global climate change, scarcity in food is going to be an imminent problem, and to meet the demands of an exploding population, the average yield needs to be increased from to 3% (von Braun 2010). In addition to the environmental atrocities, another limiting factor is the availability of nitrogen (N). Exogenous application of nitrogen fertilizer is extremely essential for most cultivated lands (Kraiser et al. 2011). In an effort to increase plant productivity, application of fertilizers has also increased drastically (Godfray et al. 2010). It has been observed that only 30–50% of applied nitrogen was converted into grains, while the rest was lost in the environment either through leaching or dissipation into the atmosphere. But the excessive use of N fertilizer has increased environmental pollution like air/water pollution (Liu et al. 2010, 2013). Consequently, it has become the need of the hour to develop crops with high ability to utilize N. N utilization can be subdivided into two processes: uptake of N (NUP), i.e., removal of N from soil by the plant as nitrate and ammonium ions, and nitrogen use efficiency (NUE), i.e., efficiency with which the nitrogen is converted to grain yield. However, the accelerated advancement in research and technology in developing crops with high N utilization means that there is an urgent requirement to develop a sustainable agricultural method (Zeigler and Mohanty 2010).

Rice is the staple food of more than half of the world's population and is a great consumer of N fertilizer (Zhou et al. 2009). Peng et al. (2006) have shown that of the 35% of global N use, in China, about 7% is used for cultivation of rice. The major expenditure incurred by rice farmers is the purchase of N fertilizer, but due to low NUE for rice, there is a loss in applied N by >50% (Shen and Zhang 2006). So reducing the application of fertilizer will not only benefit the farmers but will also positively affect the environment. Hence, many researchers have focused on developing breeding programs to introduce new rice varieties with high NUP and NUE which subsequently demands a clear understanding of the genetics of NUP and NUE in rice.

Quantitative trait locus (QTL) mapping can help in the identification of candidate genes that are key players in influencing key traits such as NUP and NUE in rice. Various studies have been carried out over the years to map QTL responsive for rice NUE. Obara et al. (2001) successfully mapped for NUE in using BILs developed from a cross between Kasalash and Nipponbare. qNUEP-6 was identified on chromosome 6 from a population of Zhenshan 97 and Minghui 63 (Shan et al. 2005). pnue9 on chromosome 9 and a QTL for NUE on chromosome 3 were identified using an RIL population from Dasanbyeo and TR22183 (Cho et al. 2007) and a DH population from IR64 and Azucena (Senthilvel et al. 2008), respectively. Several other QTLS corresponding to NUE have been identified in rice (Li et al. 2010; Wei et al. 2012). Over the years, other reports related to NUP (Piao et al. 2009) or tolerance to low level of N (Lian et al. 2005; Wei et al. 2012) or glutamine synthetase

content (Obara et al. 2004) have been published, but the genetic basis of NUP or NUE is still not well understood.

2 Molecular Breeding to Improve NUE

Nitrogen use efficiency (NUE) has been defined as the yield of the dry matter with reference to the amount of nitrogen applied and available from the soil. In other words, it is a measure of the efficiency of plants to convert the absorbed nitrogen to grain yield. Various researches have been conducted over the years on several different cultivars where higher amount of nitrogen is applied to the soil to eliminate variability in nitrogen, but it simultaneously masks the difference in efficiencies of the cultivars in absorbing and utilizing nitrogen toward grain development. Conventional breeding methodologies have been applied toward identification of the most pertinent yield-related trait or other physiological characteristics that are ultimately related to productivity.

2.1 DNA Molecular Markers

Lately, the implementation of molecular markers in plant breeding has created great potential in many applications including enhancing biotic resistance (insects and diseases) and abiotic strength (drought, low nitrogen fertilization, frost, flooding) in commercial crops. DNA molecular markers function by uncovering deviations or mutations in the specific sequence that does not affect the plant's phenotype. These markers are routinely used to identify any potential polymorphisms or DNA differences in a studied sample set or population. Polymorphisms occur due to a number of factors, but the most common type is associated with a simple insertion or deletion of a small DNA segment or few base-pair changes to the main sequence. The robustness and accessibility of DNA molecular markers and associated marker maps have yielded great success in marker-assisted selection, positional cloning of resistance genes, and mapping of entire quantitative trait loci (QTLs) in many crop applications. Restriction fragment length polymorphisms (RFLPs), Random Amplified Polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), and simple sequence repeats (SSRs) are the most widely used molecular markers in plant breeding.

2.2 RFLPs

Restriction fragment length polymorphisms (RFLPs) were the initial DNA genetic markers and were first theorized based on the technique published by Southern. First RFLPs were used by Paterson et al. in genomic searches for QTL in tomatoes. These molecular markers have the main application and specificity of locating complex traits in a plan genome due to their inherent characteristics of established existence in

plants, ability to be observed at nearly every stage of plant development, existence in nearly all plant tissues, usage without interference with targeted trait, ability to be unaffected by environmental stressors, and general robustness. Additionally, these molecular markers exhibit Mendelian codominant behavior which is essential to their ideal usage in genomics.

The use of RFLPs begins with the detection of RFLP where DNA is identified and digested with one or more restriction enzymes which specifically cleave the sequence at defined sites. The cleaved DNA is separated via gel electrophoresis and transferred to a blotting membrane to be used for identification of the distinct fragment lengths. To identify the fragments on the membrane, a DNA probe is labeled for detection on the membrane. This molecular marker method requires usage of extensive research laboratory resources which make the entire process very time-consuming, tediously completed, and expensive. In 1989, Neale et al. reported that it is expected that scanning for RFLPs in parents and progeny of one cross takes a few years. This process only identifies a single locus, and to reliably identify the same locus creates great potential for this method to be used in genomic mapping. Changes in a DNA sequence are typically identified via deviations in molecular weight which are noticed from fragment movement in the gel.

The application of RFLPS in genomic and marker mapping has progressed molecular genetics research substantially and improved plant trait enhancement methods. RFLP-based marker maps have been developed for a multitude of crops species as reported in maize, wheat, rice, cotton, barley, sunflower, tomato, and sorghum.

2.3 PCR Markers

The application of polymerase chain reaction (PCR) has helped progress the use of RFLP by circumventing the obstacles associated with the hybridization step and additionally providing another genetic analysis method for crop trait enhancement. PCR techniques share four characteristics which include low DNA amount required for usage, low time commitment, enhanced specificity, and assay completion time. These markers are typically categorized into two groups: anonymous markers (no background sequence information is necessary for marker analysis), such as RAPD, AFLP, or inter-SSR, and sequence-based markers (which rely on previous sequence information), such as SSRs or sequence-tagged sites (STS). Each class has primary applications of usage with anonymous markers used to identify multiple loci, while the other class of markers are used for single-locus identification.

2.4 RAPDs

Williams et al. first reported the development of the RAPD assay and its potential benefit to plant breeding techniques. This technique begins with random primers of about ten base pairs used to amplify the DNA sequence to identify whether or not a

polymorphism has been detected. The RAPD method is very inexpensive, quick, and robust where a single RAPD primer can be used to identify multiple loci, which presents a great application in phylogenetic experiments. It has been previously reported that a linkage map of approximately 100 individuals consisted of 150–300 specific markers within 12 months, which included DNA extraction, primer detection and generation of RAPD markers, and separation analysis. Unfortunately, these primers have been described to have issues during annealing steps, and consistency of results has been called into question. The reliability and reproducibility of RAPD markers can be very efficient in laboratories with modern PCR methods, but reproducibility between different laboratories has been a consistent problem. However, the application of RAPD has been proved successful in many crop species including maize, barley, wheat, Brassica napus, and B. oleracea.

2.5 SSRs

Simple sequence repeats (SSRs) variations called microsatellites consist of only one to six base pairs. SSR markers are universal in eukaryotic genomics, and their applications in PCR technologies have significantly advanced the field. Such high levels of polymorphisms compared to RFLPs and RAPDs, accompanied with higher rate of interspersion, help facilitate microsatellites as a robust and abundant source of genetic markers. The use of microsatellites in plants was first reported by Condit and Hubbel, and their findings proposed that there is high existence of these markers in plants and animal systems. Additionally, polymorphisms of SSRs were detected in soybeans by Akkaya et al. and thus created a new application for PCR-associated markers for new plant genomic maps. From then on, microsatellites were used in many different techniques and applications including assessment of germplasm for genetic variability and specific cultivar detection in many crop species. Once validated as useful in applications such as revealing species relationships and successfully generating phylogenetic information, microsatellites could have innumerable applications in plant breeding. Additionally, SSR loci have been mapped for multiple crops. Kadam et al. (2019) identified 90 SNP loci by making use of genome-wide association study (GWAS) from a collection of 213 randomly selected rice genotypes and were able to explain the crop model parameter variation. The study showed that SNP-based crop model was advantageous in simulating the yield under any conditions.

2.6 AFLPs

First reported by Vos et al., the AFLP method is derived from the selective PCR amplification of restriction fragments from a comprehensive and digested genomic DNA sample. This technique demonstrates the reliability of RFLP markers and the robustness of PCR together. This technique, often termed DNA fingerprinting, has significantly higher multiplex ratio compared to other marker techniques. AFLP

markers can be used to quickly and cost-effectively develop a genetic linkage map from which other markers can be mapped (RFLPs and microsatellites). These markers also identify dominant markers and have been applied to many species including maize, barley and wheat, rice, and rye.

2.7 Genome Mapping

These earlier defined molecular markers facilitate the molecular segmenting of whole genomics into more specified genetic maps. For the development of a whole-genome map, the sample population must be the consequence of a cross, and the set of molecular markers separating in the offspring generation must match Mendelian genetic ratios. Correlating directly with the method utilized, different markers are identified as dominant or codominant, a distinction which is essential in choosing the ideal genomic analysis strategy. To initialize genome mapping, the correct sample set of the progenitors in the sample population must be chosen, depending on genetic divergence of the parents to optimize the heterozygosity threshold in the next generation of progeny. Additionally, the previous generation should exhibit multiple and/or distinct phenotypes so that the segregation of the traits can be analyzed through quantitative trait mapping. To differentiate the progeny groups, labels such as F_2 or BC_1, F_3, and recombinant inbred (RI) lines or doubled haploid groups (DH) are frequently used. An inbred parental generation P1, genotype represented as "AA," with a high phenotypic value of the targeted trait is crossed with a low phenotypic value line, genotype represented as "aa." The F1 progeny inherit a copy of each chromosome from each of the two parental lines and consequently heterozygous genotypes. All F1 progeny will be genetically identical with a genotype "Aa" at each locus. Most experimental designs initiate with the F1 generation.

For a backcross, the F1 progeny are then crossed with one of the previous generation lines or parental lines. Between 100 and 1000, individuals in the F2 backcross generation receive one chromosome from the F1 and one from the original parental line. At each locus, the genotype will be "AA" or "Aa," and due to the crossing over step during meiosis, or gamete production, the chromosome inherited from the F1 generation is a mixture of the original parental lines. Thus, at each locus, there is a 50% chance of inheriting the dominant parental allele and another 50% chance of inheriting the recessive parental allele. A variety of loci will be inherited into the chromosome with different amalgamations of dominant and recessive alleles. In plant breeding and genome mapping, an intercross is often used as another experimental design. The intercross consists of an F2 population made from self-mating or sibling-mating F1 progeny together. The F2 progeny inherit two sets of chromosomes from the F1 generation which consist of a mixture of the original parental chromosomes. At each locus, the F2 progeny will have genotypes of AA, Aa, and aa and can now be utilized for different types of mapping methods due to superior amount of genetic diversity. In a double haploid (DH) population, the progeny are produced from pollen on an F1 plant and cultivated through an anther

culture and by chemically inducing chromosome doubling. The DH population genotypes are homozygous (AA or aa) in various loci in the chromosome. These populations are sometimes called permanent populations due to the fact that there will be no trait or allele segregation in future progenies. This creates an advantage for genome mapping because marker data can be reused in different locations over long periods of time, making one of the most popular populations for many experimental designs. The rates of pollen that successfully yield DH progeny can fluctuate significantly which can lead to segregation distortion and incorrect pairing between marker loci as consequence.

An RIL population or recombinant inbred line can be constructed by self-mating or sibling-mating progeny for many generations. From the F2 individual, a single-seed approach is typically used until all trait loci are homozygous. Some of these populations have been successfully cultivated in rice, maize, *Arabidopsis*, etc. The distinct advantage of these population types is that their genetic distances are often significantly larger compared to previous F2 or BC populations due to the increase chance of recombination from multiple generations of self-crosses or sibling crosses. Such populations could prove very valuable in enhancing accuracy in QTL mapping. Modern computing and software programming have made massive data sets much easier to analyze, specifically for estimating recombinant frequency and locus ordering. The Haldane (1999) or Kosambi (1944) method is a mapping tool utilized in the conversion of recombination fractions into linkage map units or centiMorgan (cM). The Haldane method uses the occurrence of many crossovers in the progenitor generations, while the Kosambi method takes into account any potential interference. Many software programs that are currently used to analyze mapping data include MAPMAKER, G-Mendel, MapManager, or JoinMap.

2.8 QTLS

The relationship between a genotype and a phenotype is what is directly correlated with the analysis of classic Mendelian genetics. Any differences in phenotype among progenitors in a generation are directly related to their genotype at the single locus. Additionally, most traits of commercial interest in crops are not associated with distinct phenotypic classes, but are the consequence of the cooperative action of many genes demonstrating quantitative variety. Polymorphic single genes are utilized to initiate plant breeding as reported by Sax. Sax reported that there was a distinct linkage between seed size in *Phaseolus vulgaris* (quantitative trait) and seed color (qualitative trait). Thoday reported that single-gene markers are utilized to comprehensively characterize and map genes with individual Mendelian factors that are associated with quantitative traits. During this period, the accessibility of adequate genetic markers was significantly restricted. Now, complete and comprehensive genetic molecular marker maps for many major commercial crops and associated analysis algorithms have been specifically developed for QTL mapping. Modern advances in the last few decades have revolutionized plant breeding in many crops using molecular markers to track and express sought-after genes. The use of

the Bt transgene into multiple applications is a prime representation of the successful transfer of a single target region. Paterson reported comprehensive reviews of the method and the utilization of molecular markers to assist plant breeding. Liu reported the progression of computing and software analyses associated with molecular markers with single or multiple traits, while Powell et al. reported the differences between the beneficial and undesirable characteristics of molecular marker methods. By utilizing markers in plant breeding, breeders have been able to move away from the outdated breeding method of phenotypic selection. A plant's phenotype is related directly to its genotype and the environment surrounding its proliferation where environmental factors can disguise some genotypic traits. Consequently, breeding scientists must estimate a plant's genetic potential to select progenitors that do not express advantageous traits. Molecular markers help breeders facilitate enhanced selection associated with genotypic or DNA-based variations rather than phenotypes to enhance selection efficacy. In most major crop species, high- to mid-density molecular maps encompassing whole genomes have existed for many years. Using chromosome segment substitution lines (CSSLs) developed from a cross between 9311 (recipient) and Nipponbare (donor), Zhou et al. (2017) identified seven and six QTLs for NUP and NUE, respectively. In addition, they also identified QTLs for biomass yield (BY) and grain yield (GY). They also observed that GY correlated positively with NUP and NUE. In another study carried out by Han et al. (2016), they identified and mapped candidate genes from barley-based genetic homology and expression analysis. For crops like rice, identification of QTLs for a complex trait like NUE is exceedingly difficult as it is associated with different components like pNUE, aNUE, agNUE, and other agro-morphological traits like grain yield, plant height, tiller number, filled grains per panicle, grain weight, etc. (Ali et al. 2018). *qRDWN6* conferred tolerance to N deficiency in rice and was identified on the long arm of chromosome 6 using a CSSL population. It was identified near marker InD90 based on association analysis of phenotypic data and polymorphic markers. Further, they narrowed down the candidate region to 52.3-kb spanning the markers ND-4 and RM19771 which comprised of nine candidate genes. One of these was a potassium transporter that is predicted to be the strongest candidate (Anis et al. 2019).

Molecular markers are not associated directly with a distinct phenotype and are used to map both dominant and recessive inherited traits controlled at a single-locus (qualitative traits) and multiple loci-controlled traits (quantitative traits). Quantitative traits inherited physical characteristics such as height, crop yield, disease and insect resistance, stress tolerance, and consistent disparity in phenotypic manifestation. In the last few years, quantitative traits were solely researched using quantitative statistical analysis methods in experimental populations. These methods were utilized to observe the intricacy of quantitative traits and to understand the individual loci's genetic effects. Molecular markers have the distinct benefit in the application of isolating quantitative traits into genetic loci allowing the effects of a single locus to be observed.

Quantitative trait loci (QTL) mapping can be described in five levels of increasing intricacy. The initial step is a single-marker analysis which is an assessment of the relationship between trait values and the associated genotypes of marker loci. This assessment assesses each marker locus individually, without the prerequisite that the marker loci be mapped in relation to one another. In this technique, each locus is assessed for its relationship by multiple regressions blended with a consistent group of background markers. These two most common techniques have been utilized to approximate the regression: least squares regression and maximum likelihood estimation. One important setback associated with single-marker analysis is that this technique cannot differentiate between distinct linkage to a QTL with minimal effect and indistinct linkage to a QTL with a significant effect. The secondary level of QTL mapping is known as simple interval mapping (SIM) and necessitates prior construction of a marker genetic map. The SIM method explores a single target QTL throughout an entire mapped genome. The expected QTL genotype is approximated from the known genotypes of flanking marker loci and their respective distance from specified QTL. The analysis point that produces superior association may be understood as the alleged location of the targeted QTL. When multiple QTLs segregate, the sampling error related with identification of a QTL may be increased by interference of other QTLs, and additionally, linked QTLs can cause biased approximations of position of a loci. Several techniques fitting multiple QTLs have been suggested which function to quantify to the third level of QTL analysis, composite interval mapping (CIM), or multiple QTL mapping (MQM). These CIM methods facilitate mapping in one of two ways, related directly to whether the background marker and the target interval are associated. If they are not linked, inclusion of the background marker makes the analysis more profound to the existence of a QTL in the specified interval. If they are linked, inclusion of the background marker may help to isolate the target QTL from other linked QTLs on the far side of the background marker. The fourth level adds automatic background marker selection to CIM. Two programs (QTL Cartographer and PLABQTL) function with stepwise regression to detect eligible background markers; others use simple statistical regression. MQTL is a software package that utilizes a basic form of composite interval mapping for QTLs in massive data sets consequent from multiple environments. Like PLABQTL, it will approximate environmental effects and QTL-environment interactions. A fifth level is an unconventional approach, which is automatic construction of multi-QTL models by Bayesian methods. These methods necessitate orders of magnitude more computation than those described above, but they provide a way to consider automatically many combinations of QTLs, QTL positions, and QTL strengths. Moreover, what is even the most challenging of these techniques is that these are cost-effective when likened against the cost of obtaining marker data. Specialized QTL software packages that analyze specific genetic and statistical methods have been developed mainly by scientists who are working in the area of statistical genetics, such as MAPMAKER/QTL, QTL Cartographer, MAPQTL 4.0, MapManager QTX, MQTL, and QGENE.

2.9 Marker-Aided Selection (MAS)

The plant breeding field has recently progressed toward marker-aided selection (MAS) instead of more traditional methods. Many traits are economically relevant including grain or forage yield and can be categorized as multigenic or quantitative. Some traits that are simply inherited, such as disease resistance, can be described as "semiquantitative", when allele expression is controlled by several genes (e.g., a major gene plus several modifiers). Although plant breeding programs are significantly concentrated on improving crop yield, the yield must be sufficient for consumer usage. Therefore, traits such as standability (or lodging resistance), disease resistance, and insect resistance must also be sought after for commercial crops. Marker-based methodology has recently provided breeding scientists with a potent approach for detecting and mapping QTLs and may eventually lead to a more comprehensive understanding of genetic phenomena such as epistasis, pleiotropy, and heterosis. It must be recognized, however, that methods such as these have detected and mapped only rather large chromosomal segments (in most cases probably 20–30 cm long). Although results from such publications may be sufficient for many plant breeding activities, novel methods will be essential to detect individual genes and quantify individual gene action and interactions between other genes. Nearly every trait that is agronomically or economically relevant was exposed to DNA marker mapping and QTL analysis. Molecular markers have been utilized to map genes for drought tolerance, seed hardness, seed size, maturity and plant height, disease resistance, oil and protein content, soluble solids, heterosis, insect resistance, and yield. Studying plant tolerance and resistance to abiotic stresses will help facilitate detection of genes associated with these traits, and the molecular markers can simplify understanding of the genetic basis of resistance and the impact of individual QTLs on its phenotypic appearance. Plant and animal breeders do not necessarily need to know the distinct locations of their QTL with significant accuracy if they intend to cross them by marker-assisted backcrossing. They will be mainly focused on those QTL which exhibit a significant phenotypic effect. These novel methods will also help breeders to regulate the ideal ideotype from QTL studies of many crosses and facilitate the opportunity of constructing them. Probably the most significant value of markers in these methods is in the decrease of linkage drag during introgression of QTL by backcrossing. Inversely, map-based gene cloning of QTL necessitates somewhat more significant mapping accuracy than is currently commercially accessible.

2.10 Future of MAS

The proportion of QTL detected using rationally strict criteria are real and heritable contributors to genetic variation, as found in recent advances in plant breeding studies. Experimental tests of the efficacy of MAS are very significant in plant breeding research. Lande and Thompson reported that a single trait has high potential selection efficiency by using an amalgamation of molecular and phenotypic

information, correlating to traditional methods of phenotypic selection, contingent on the inheritability of the trait, the proportion of additive genetic variance associated with the marker loci, and the selection methodology. Marker-assisted introgression of genes between maize lines has been successfully proven to produce higher yield in hybrid cultivars, although no controls were utilized in these studies to observe success without marker information. MAS for salt tolerance improvement for tomato cultivar populations has shown efficacy. Analytical methods reported by Lande and Thompson were concentrated on first progeny selection. Succeeding modeling studies were concentrated on the efficacy of MAS in applications over multiple successive generations using computer simulation software. The results from these studies demonstrated significantly that MAS could be more efficacious than traditional phenotypic-based selection in massive populations and for specific traits with inherently low inheritability. The computer simulations additionally demonstrated that supplementary genetic gain provided by MAS, when linked with traditional phenotypic selection, quickly lowered after many successive cycles of selection and that in the future MAS may potentially become less efficient than phenotypic-based selection. Considering that when the effects associated with markers are not reevaluated at each generation, this issue becomes much more serious. Other reported developments in maize failed to establish response to MAS. Nevertheless, in both of these situations, there was insignificant evidence for response based on phenotypic selection. Additionally, population sizes were kept small with no replicates. On the other hand, more convincing evidence for potential utility of QTL information has arisen from several reasonably convincing studies in major commercial crops, indicating that a significant proportion of QTL can be established across different environments and progenitor generations and among independent traits. Additionally, the addition of marker information has successfully demonstrated the ability to increase precision of prediction in later generations' performance in maize compared to F2 phenotype information by itself. Recently, more noteworthy examples with almost no QTL were established in independent experiments with the same population. The genetic architecture of population variance has persisted as a significant enigma in the progression of quantitative genetics. Most recently, research has been focused on uncovering the origins of heterosis and whether or not the collaboration among loci is a general phenomenon. Mounting evidence in recent research has depicted the primary cause of heterosis being dominant rather than overdominant. However, experiments in tomato and maize have reported significant overdominant action of some QTLs, while a few of the seemingly overdominant QTL in maize were afterward analyzed comprehensively, and each was validated to be partially dominant loci in repulsion phase linkage disequilibrium. The boundless number of potential questions and limits to precision of individual results will guarantee that molecular genetics has an important role in quantitative genetic and plant breeding research in the foreseeable future. Before MAS commitment can be solidified in the industry, QTLs that have been identified must be validated in many other different experiments, as well as in varying genetic and environmental conditions. Yet, the viability of identifying QTLs in commercial crops has previously been successfully established, and most

plant scientists have voiced their approval and pledged their future research toward the progression of MAS. Large mapping populations are required for a more accurate approximation of the scale of QTL effects and for identification of QTLs with less than significant effect. More validation-focused experiments are also required to demonstrate the biological potential of QTLs. Moreover, until more validated date is reported about all of the potential differences in temporal, environmental, and genetic stability of QTLs, MAS is unfortunately limited to the same genetic and environmental conditions in which the QTLs were originally identified.

3 How to Identify Genes Controlling NUE

NUE is a complex polygenic trait, and identification of genes controlling it requires quantitative genetics, and the initial step in the process is QTL identification. Recently, a lot of progress has been made in QTL identification through development of statistical analysis and mapping populations. The process is, however, reliant on two primary questions: (1) Is the analysis being carried out to decipher the genetic mechanism or traits associated with NUE? (2) Does it focus on the identification of new candidate gene(s)/alleles and simultaneously the mechanism from different germplasms? However, one thing that is essential to consider here is that each approach has its own positives and negatives.

4 Association Genetics

Plant genotypic variation based on the environment, allelic variation proves very effective via association genetics in providing a high resolution genetic map reliant on recombination event and have been successfully implemented in researches related to NUE (Cormier et al. 2014). Statistical analysis for the association genetics cannot be performed with low confidence due to low heritability of the NUE traits. The problem can be solved by increasing the size of the sample, but this leads to the increase in the expense of the experiments. Another important problem that arises in this regard is that in an effort to increase the statistical stringency, low-frequency allele gets removed ($<10\%$) that essentially come from different landraces.

5 Role of Phenology in Gene Discovery

Phenology refers to the different stages of plant development from germination to senescence. However, the phenology and hence the physiological aspects of plant development is affected by different environmental factors ranging from soil salinity to extremes of temperature. Hence, it becomes essential to optimize the phenological profile as it has been shown to correlate with NUE and the identified QTL for phenology traits collocates with NUE which is probably linked through pleiotropy. It is always beneficial to have a study conducted for large association panel of

genotypes where if a phenology like increase in flowering time is observed it can be validated essentially with NILs and where if the same phenology is observed as one parent it can be easily attributed to NUE rather than a consequence of environmental adaptation. It needs to be mentioned here that fine-tuning of phenological traits like selection of genotypes that are well adapted to specific environment for floret abortion [49] improved NUE be achieved. An essential problem with NILs is that de novo QTL for NUE still needs to be conducted. This problem can be solved by making use of backcross and CSSL populations, but it needs a large sample size for scanning a single genome. An alternative for this can be making use of multiparent populations that will comprise of a large sample set. To conclude, it can be said that if a large data set for phenological traits are available, it becomes possible to minimize the confounding effects of identifying QTLs for NUE.

6 Looking Toward the Future to Improve Crop Productivity

In the coming years, the major target for researchers more specifically the breeders or the farmers is to improve the yield. The major focus in this regard is to develop cereal crops with high ability to capture N via the root system so that the low worldwide N use efficiency can be overcome (Raun and Johnson 1999). A well-organized agronomic studies can be highly helpful in implementing this, and a lot for focus needs to be given on improving root traits as there are still scope for improvement. Researches show that targeting the photosynthetic and structural traits can be helpful in improving the yield and NUE per say but it might lead to compromise the quality. Hence, it will be nice to focus on improving NUE targeted toward effective conversion of N to grain. To achieve this, researchers need to focus on the widely available rice germplasm resources that exhibit a lot of genetic variation and can contribute toward identification of novel alleles. A limiting factor in this regard that to perform the screening experiments for such widely variant sample size is that the field trial needs to be conducted in different locations and for several years and also needs greater expertise in phenology and a clear understanding of NUE parameters. These problems can be undermined by use of optical sensors (Erdle et al. 2011) or NDVI (normalized difference vegetation index) (Aparicio et al. 2000). Recent advancement in technology has brought in automated mobile and fixed platforms for efficient measurement of these parameters both temporally and spatially (Andrade-Sanchez et al. 2014; Virlet et al. 2017).

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Improving Water Use Efficiency and Nitrogen Use Efficiency in Rice Through Breeding and Genomics Approaches

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Abstract

Rice is a staple food of more than half of the world's population; more than 3.5 billion inhabitants depend on rice for obtaining 20% of their daily calorie intake. Nitrogen is the most important for crop growth and yield potential. Indeed, nitrogen is essential to stimulate tillering, leaf growth, photosynthesis, and protein synthesis. Significant achievements have recently been observed at the molecular level in nitrogen use efficiency and water use efficiency in plants. In this chapter we will discuss the following issue: (i) definition of both nitrogen use efficiency and water use efficiency, (ii) genes responsible for nitrogen use efficiency and water use efficiency, (iii) best ways for improving water and nutrient use efficiency in rice, and (iv) optimizing nitrogen options for improving water and nitrogen use efficiency of rice under different water regimes.

Keywords

Rice · Water use efficiency · Nitrogen use efficiency · Breeding · Genomics approaches

1 Nitrogen Use Efficiency (NUE)

NUE is definite as the proportion of grain yield to the provided nitrogen (N) (Shi et al. 2010). It has collected of two primary components like the nitrogen uptake efficiency (NUpE) and nitrogen utilization efficiency (NUtE) (Haefele et al. 2008). NUE is defined as the ratio of plant nitrogen content to the accessible nitrogen content, and NUtE is similarly defined as the ratio of grain yield to the available plant nitrogen content (Moll et al. 1982). Similarly, NUtE was called physiological NUE (Singh et al. 1998). Nitrogen fertility is a significant component of rice (*Oryza sativa* L.) cultivation systems.

Most of the nitrogenous fertilizer is lost due to gaseous emission, outward runoff volatilization, and leaching and lastly enters the environment. There is a solid impact of nitrogen on plant growth, so farmers supply huge amount of nitrogen fertilizer in

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order to get maximum productivity, but plants usually consume a smaller amount than half of the nitrogen applied in their fields, and a major portion of it is lost to the environment or leached into several water bodies causing severe environmental pollution (Shi et al. 2010). NUE is comparatively low in irrigated lowland rice system because applied inorganic N is quickly lost from the soil flood water system by volatilization and denitrification (De-Datta and Buresh 1989). It was exposed that agronomic NUE (kg grain yield increase per kg of nitrogen applied) of rice was 15–18 kg N ha^{-1} in the dry season in the Philippines (Cassman et al. 1996). It was assessed that reducing nitrogen fertilizer input could significantly improve NUtE (Peng et al. 2010). It was conveyed that there is higher NUE in grain yield of hybrid varieties than the conventional varieties. It was also estimated that in southeastern China the economically optimal and ecologically optimum nitrogen rates for the rice production were 180–285 kg ha^{-1} and 90–150 kg ha^{-1} resulting to rice yields of 6.1–8.9 t ha^{-1} and 5.5–8.8 t ha^{-1}, respectively (Chen et al. 2011). Ladha et al. (1998) discoursed that desirable cultivars with high NUE produce large yield nevertheless of the nitrogen supply as grain yield and total *N* uptake were significantly affected by varieties. To attain both high yield and efficiency of fertilizer application, it is suggested to enhance fertilizer management at critical growing stages (Qiao et al. 2012). It was initiated that in northeast Thailand, grain yield was 4 t ha^{-1} when *N* is applied at 40, 80, and 90 kg ha^{-1} at panicle initiation, heading, and maturity, respectively (Ohnishi et al. 1999).

2 Water Use Efficiency (WUE)

Breeding to report explicit objective implies first that the objective has been well defined and, second, that heritable traits have been recognized that can come some way to achieving the breeding objective. WUE as a breeding target could be defined in numerous ways, dependent on the scale of extent and the units of exchange being considered. All potential definitions will have some measure of water being exchanged for some unit of production. For physiologists, the basic unit of production could be moles of carbon gained in photosynthesis (A) in exchange for water used in transpiration (T). Consequently, a physiological definition might liken, at its most basic level, to the instantaneous WUE of leaf gas exchange (A/T). For an agronomists and farmers, the unit of production is much more likely to be the yield of harvested product achieved from the water made available to the crop through precipitation and/or irrigation, such as farmer's definition is one of agronomic WUE.

Agronomic WUE will be taken to be the ultimate breeding target. To do this it is beneficial to consider crop yield as being constructed from a framework of relatively simple components (Eq. 1).

$$\text{Yield} = \text{ET} \times \text{T/ET} \times \text{W} \times \text{HI} \tag{1}$$

In this context, grain yield is described as being a function of the amount of water used by the crops (evapotranspiration, ET), the amount of that water really transpired

by the crop (T/ET), and the transpiration efficiency of biomass production (W), i.e., how much biomass is produced per millimeter of water transpired and, finally, how effectively the achieved biomass is partitioned into the harvested product, i.e., the share of grain yield to standing biomass labeled the harvest index (HI). This is not based on the concept of "drought resistance" but rather on the broad processes by which crops actually achieve yield in water-limited environments (Passioura 1977; Condon and Richards 1993; Richards et al. 2002). None of the components of this yield is truly independent of the others (Condon and Richards 1993), but each can be measured a target for inherent improvement. Leaf-level WUE, A/T, is directly related to only one of these components, W, the transpiration efficiency of biomass production. However, as will be discussed in following sections, A/T also has the potential to influence each of the other three components in the yield framework.

Increasing the WUE of both irrigated and rainfed crop production is an important imperious (Hamdy et al. 2003). Of the world's allocable water resource, 80% is currently consumed by irrigated agriculture. Anticipated population growth (another 2 billion people within two to three decades) will need that more of the available water resource be used for domestic, municipal, industrial, and environmental needs. The most realistic resolution to the improved demand for water will be reallocation to these other purposes of some of the water currently used by agriculture. Even a modest reallocation, dropping agriculture's share to 70%, would increase the amount of water available for other purposes by up to 50% (Hamdy et al. 2003). However, the increased populations will not only need more water to satisfy these other purposes; it will also need to be nourished and clothed. This will require substantially more efficient production from a lesser irrigation water resource. It will also require substantially higher WUE from rainfed agriculture, which remains the key means of food production in most countries and for most farmers. Numerous plans will be mandatory to improve the productivity of water use in irrigated and rainfed agriculture (Wang et al. 2002). Breeding crop varieties that are more efficient in their water use is one such approach. Others comprise better management of the water resource and fluctuations in crop management. None of these strategies should be seen as operating in isolation. Relatively, it is expected that the highest gains will be obtained through complementary approaches involving each of them.

Rice is a key user of agricultural inputs in India, including water. Based on hydrological regimes, soils, and climatic conditions, rice production systems are classified into six different categories (Prasad et al. 2012), viz., irrigated (wet season), irrigated (dry season), rainfed upland, rainfed lowland, deep water, and coastal lowland production systems. Irrigated rice production system covers about 44%, while rainfed production system covers about 45% of the worldwide rice area. The accessibility of water, even though it is a renewable natural resource, is seriously reliant on the productivity of the source (river, canal, and pond) and the distribution system prevalent in a country. In case of groundwater as the source, availability of water is governed by the availability of electricity. Quality of water is similarly a concern in most of the regions. Satisfactory water availability and its easy access are major issues determining rice yields, and numerous researchers have reported its requirement (Sharma et al. 2002; Chauhan et al. 2012). Serious concern has been

elevated about the exhaustion of surface water resources, reducing levels of groundwater (Barker et al. 1999) and lowering of water table due to rice cultivation in India (Hira et al. 2004; Jalota et al. 2006; Hira and Jalota 2009; Kumar et al. 2010).

3 Genes Responsible for NUE and WUE

Significant achievements have recently been observed at the molecular level in NUE and WUE in plants. But progress made so far for explication of various traits that contribute to NUE and WUE is not entirely understood and is being revisited. Molecular genetics is important for identification and characterization of genes that play a vital role in increasing plant NUE and WUE. WUE refers to the ratio of water used in plant metabolism to loss of water through transpiration. Generally, two types of WUE are referred: photosynthetic WUE (also known as instantaneous WUE), which is the ratio of the rate of carbon assimilation to transpiration rate, and WUE of productivity (also known as integrated WUE), which is the ratio of production of biomass to transpiration rate (Hall 2005). WUE aids in decreasing competition for water, increasing plant productivity, and surviving periods of drought stress and therefore contributes to sustainability of water (Evans and Sadler 2008). Plant NUE is a complex process, with each step being governed by a network of multiple genes and their interactivity with environmental factors. Plant NUE is estimated by plant seed yield relative to the amount of nitrogen applied and is generally a combination of NUpE and NUtE (Moll et al. 1982). There is a complex regulation of nitrogen uptake, assimilation, and remobilization. Modifying plants genetically and implementing coordinated agricultural management will lead to enhancement of plant NUE. For successful amelioration of NUE, the former is an effectual biotechnological method. To accomplish this, manipulation of specific genes that maintain the balance of nitrogen and carbon metabolism and overexpression of nitrate (NO_3^-) and ammonium (NH_4^+) transporters responsible for N uptake by roots are a prerequisite. Processes including nitrogen acquisition, assimilation, and remobilization are essential for improving plant NUE. Therefore studies have focused mainly on the genetic manipulation of these processes and their regulation to improve plant NUE (Yang et al. 2017).

3.1 Nitrogen Acquisition

Nitrogen can be acquired by plants through their roots from the soil in inorganic forms as NO_3^- and NH_4^+ and organic forms as urea, amino acids, and peptides. Even though organic forms promote plant N nutrition in specific habitats (Taiga biomes), NO_3^- and NH_4^+ are the universal forms in most soils. Their availability in natural soils is generally low but can also vary differently depending on various factors including soil physical properties, leaching, and microbial activity, which often result in N depletion in the soil (Kiba and Krapp 2016). In transporters that mediate uptake of N at low nitrogen concentrations, less than 1 mM are known as

high-affinity transportation systems (HATS), while in transporters which mediate N uptake at high N concentrations, more than 1 mM are known as low-affinity transportation systems (LATS) (Miller et al. 2007; Williams and Miller 2001). Four families of transporters, NO_3^- transporter 1/peptide transporter family (NPF, also referred as the NRT1/PTR family), NO_3^- transporter 2 family (NRT2), the chloride channel family, and slow anion channel-associated homologues, have been identified for NO_3^- uptake (Kiba and Krapp 2016). In rice, uptake and translocation of NO_3^- is mediated by two families, NPF and NRT2 (or NAR2/NRT2). Most of the NPF family members described to date were low-affinity NO_3^- transporters, with the exception of OsNPF6.5 (NRT1.1b) which shows dual affinity of NO_3^- transportation behavior associated with increased NO_3^- absorption and root-to-shoot transportation. OsNPF6.5, considered an OsNPF8.9 (NRT1/NRT1.1/NRT1.1a) putative mRNA splicing product, has significant effects on both NUE and yield (Huang et al. 2018). OsNPF8.9, expressed primarily in root epidermis and hairs, was cloned to contribute to N uptake (Li et al. 2017). Fang et al. (2013) demonstrated the role of OsNPF4.1 (SP1) in rice panicle elongation and the function of OsNPF8.20 (OsPTR9) in NH_4^+ uptake, lateral root formation, and increased grain yield (Fang et al. 2013). Their substrates, however, are still unknown. To date, seven genes of NRT2 have been identified in Arabidopsis from which AtNRT2.1, AtNRT2.2, AtNRT2.4, and AtNRT2.5 are expressed in the roots of N-deprived plants. Research of a quadruple mutant found that these four NRT2 transporters account for about 95% of N-restricted high-affinity NO_3^- influx activity, with AtNRT2.1 being the major contributor (Lezhneva et al. 2014). Five genes encoding HATS components (OsNRT2.1/2.2/2.3a/2.3b/2.4) and two NAR2s (OsNAR2.1/2.2) have been identified in rice, each with different patterns of expression and regulation (Kiba and Krapp 2016). OsNRT2.1 and OsNRT2.2 share a similar sequence of coding regions with separate 5′ and 3′ untranscribed regions among the five OsNRT2 genes. Alternative splicing of OsNRT2.3 gives two derivatives, OsNRT2.3a and OsNRT2.3b. OsNRT2.3a is expressed mainly in the xylem parenchyma of root, whereas OsNRT2.3b is expressed predominantly in the pH-sensitive shoot phloem (Feng et al. 2011). OsNRT2.3a helped in long-distance transport of NO_3^- at low NO_3^- concentrations from root to shoot, and OsNRT2.3b increased N, Fe, and P grain uptake and improved grain yield and NUE (Araki and Hasegawa 2006). The primary and lateral roots showed abundant expression of OsNAR2.1, OsNRT2.1, and OsNRT2.2. Overexpression of the OsNRT2.1 gene alone did not increase NO_3^- absorption in rice because OsNRT2.1, OsNRT2.2, and OsNRT2.3a need a partner protein, OsNAR2.1 (Feng et al. 2011).

Proteins of ammonia transportation protein (AMT)/transportation of methyl ammonium/rhesus superfamily mediate NH_4^+ uptake (Kiba and Krapp 2016). The intervention of AMT members plays a more prominent role in NUpE in rice which favors NH_4^+ than in crops that use nitrate. In rice, there are at least ten putative OsAMT-like genes grouped into four subfamilies (i.e., three respectively for OsAMT1, OsAMT2, and OsAMT3 and one for OsAMT4) (Yang et al. 2017). Studies on the control of expression of AMT genes in rice so far focus primarily on the OsAMT1 gene family, which showed different patterns of spatio-temporal

expression in response to changes in N levels or regular irradiance (Li et al. 2017). OsAMT1;1 plays a major role in NK homeostasis, while OsAMT1;2 can serve as a nitrogen assimilator, and OsAMT1;3 acts as a nitrogen sensor (Potel et al. 2009; Kiba and Krapp 2016; Yang et al. 2017; Huang et al. 2018).

3.2 Nitrogen Assimilation

Until incorporation into organic form, nitrite reductase (NiR) first reduces all inorganic nitrogen to ammonia in plastids, and ammonia is then assimilated into glutamine (Gln) and glutamate (Glu), which translocates organic nitrogen from sources to sinks. The major enzymes involved are glutamine synthetase (GS), glutamate synthase (GOGAT, aminotransferase glutamine-2-oxoglutarate), and glutamate dehydrogenase (GDH) (Lam et al. 1996). GOGAT catalyzes the transition to 2-oxoglutarate (2-OG) of the amide group of Gln formed by GS to yield two Glu molecules. One of the Gln molecules as a substratum for the GS reaction is cycled back, and the other can be used for many chemical reactions (Masclaux-Daubresse et al. 2006). Rice has three homologous yet distinct GS genes (i.e., OsGS1;1, OsGS1;2, and OsGS1;3) and one chloroplastic gene (OsGS2). OsGS1;1 and OsGS1;2 both demonstrated a high NH_4^+ substrate affinity and were stimulated by NH_4^+ within the rice-elongating zone's central cylinder. OsGS1;1 was expressed in a constitutive way with a higher expression in leaf blade and was involved in natural rice growth and grain filling (Tabuchi et al. 2005). GOGAT also has a small family of genes: one type dependent on ferredoxin (Fd) and two types dependent on nicotinamide adenine dinucleotide hydrogen (NADH). OsFd-GOGAT is highly abundant in light-regulated mesophyll cells and other chloroplast-containing cells and is essential in chloroplasts for the re-assimilation of NH_4^+ formed by photorespiration (Xu et al. 2012). It has recently been reported to be involved in C/N balance, leaf senescence, nitrogen assimilation, and nitrogen remobilization. OsNADH-GOGAT1 is expressed primarily in $NH4^+$-dependent surface cells of rice roots and is essential for the primary assimilation of NH_4^+ in roots during the seedling stage and the production of the effective tiller number until harvest (Li et al. 2017). OsNADH-GOGAT2 is primarily expressed in mature leaf blade vascular tissues and is important in the process of Gln generation in senescent leaves to remove leaf nitrogen from the panicle during natural senescence. Mutants from OsNADH-GOGAT2 had a substantial decrease in the number of spikelets per panicle (Masclaux-Daubresse et al. 2010). In addition to the GS/GOGAT process, two enzymes are likely to be involved in the assimilation of NH_4^+: asparagine synthetase (AS) and carbamoyl phosphate synthase (CPSase). Cytosolic AS catalyzes the amido glutamine group's ATP-dependent transition to an aspartate molecule to generate Glu and Asn. Masclaux-Daubresse et al. (2006) provided evidence that ammonia may also be used as a substrate by AS. Three genes encode AS in Arabidopsis (ASN1, ASN2, and ASN3). Asn has a higher N/C ratio than Gln and can be used, especially in legumes, as a long-range transport and storage compound (Lam et al. 2003). In some situations, AS could compensate for the reduced

assimilatory activity of GS-dependent NH_4^+. CPSase forms carbamoyl phosphate, a citrulline and arginine precursor, in plastids that use bicarbonate, adenosine triphosphate (ATP) and NH_4^+, or the amide glutamine group. carA and carB are two genes encoding CPSase. Single copy of carA encodes the smaller subunit, and carB encodes the larger subunit of CPSase to form a single heterodimeric enzyme in Arabidopsis (Potel et al. 2009). NR, NiR, and GOGAT require enzyme-based power reduction either as NADH or Fdx. ATP is required by GS and AS.

3.3 Nitrogen Remobilization and Assimilation

Remobilization of nitrogen is essential for production of seeds and nitrogen content of seeds. Nitrogen content influences the germination efficacy and survival of young seedlings. Nitrogen remobilization has been studied in several plant species. The estimation of the total amount of nitrogen present in the different plant organs at different times of growth and the long-term labeling of 15 N allow fluxes to be determined (Gallais et al. 2006). N remobilization is an environment-dependent process and occurs generally during limited NO_3^- supplies (Masclaux-Daubresse et al. 2010). It has been shown in Arabidopsis and oilseed rape that nitrogen can be extracted at the reproductive stage from senescence leaves to expanding leaves at the vegetative stage and from senescence leaves to seeds (Diaz et al. 2004). The leaves are sink for N during the vegetative stage; later, during senescence, this N is removable for reuse in the seeds, primarily as amino acids (Okumoto and Pilot 2011). Gln and asparagine (Asn) are important sources of total amino acids in phloem and xylem sap of rice plants (Li et al. 2017). Increase in both Asn and Gln concentrations in the phloem sap during senescence indicates their main role in making N available from senescing leaves for remobilization. During N remobilization (Huang et al. 2018), certain GS1, NADH-GDH, and AS isoforms were strongly triggered. The existence of amino acid transporters, which belong to diverse multigene families, is not well known in phloem loading during senescence for N redistribution (Perchlik and Tegeder 2017).

OsGS1;1 and OsNADH-GOGAT2 are essential genes during natural senescence for remobilization of nitrogen. In the production of active tillers, GS1;2 is also important through the assimilation of $NH4^+$ produced during lignin synthesis. It is assumed that AS plays a crucial role in primary N metabolism together with GS, catalyzing Gln and aspartate formation of Asn and Glu (Masclaux-Daubresse et al. 2010). There are two genes that encode AS in rice, i.e., OsAS1 and OsAS2. OsAS1 is expressed predominantly in $NH4^+$-dependent way in root surfaces (epidermis, exodermis, and sclerenchyma), which are very similar in rice roots to OsGS1;2 and NADH-GOGAT1. Therefore, AS1 tends to be associated with the primary assimilation of $NH4^+$ in rice roots. OsAS2 is found abundantly in phloem companion cells and parenchyma cells of leaf blades and sheaths along with GS1;1 protein (Huang et al. 2018). These suggest that AS2 in rice leaves is likely essential during natural senescence in long-distance Asn transport from rice leaves. Also, the mitochondrial GDH plays a vital role in the re-assimilation of photorespiratory ammonia and can

alternatively incorporate NH_4^+ in Glu as a response to high levels of stress (Masclaux-Daubresse et al. 2006). Although a large number of amino acid permeases (AAPs) are identified in rice, no transporters have been functionally characterized with the exception of OsAAP6, which is expressed mainly for grain protein content in seeds (Peng et al. 2014). Taylor et al. (2015) studied the transportation role of four rice AAP genes (OsAAP1, OsAAP3, OsAAP7, and OsAAP16) in *Xenopus laevis* oocytes, cell position, and electrophysiology. OsAAP1, OsAAP7, and OsAAP16 are general AAPs and could well transport all amino acids except aspartate and β-alanine, although OsAAP3 had a clear specificity of the substrate which transported the basic amino acids lysine and arginine well only when selected against aromatic amino acids (Perchlik and Tegeder 2017). Increasing nitrogen remobilization is useful in reusing nitrogen from the vegetative parts and curtailing nitrogen loss in the dry remains.

4 Improving Water and Nutrient Use Efficiency in Rice

Nutrient use efficiency can be defined in multiple ways, but the most commonly used definition by farmers and crop advisers is the crop output per unit of nutrient input. We are all interested in improving the efficiency with which we can simultaneously maximize productivity, profitability, sustainability, and environmental protection. WUE refers to the ratio of water used in plant metabolism to water lost by the plant through transpiration. As per FAO WUE is the ratio between effective water use and actual water withdrawal. It characterizes, in a specific process, how effective is the water utilization.

Rice is a staple food of more than half of the world's population; more than 3.5 billion inhabitants depend on rice for obtaining 20% of their daily calorie intake (IRRI, Africa Rice, and CIAT 2010). The global population is increasing in geometrical progression. So, to meet the demand for food, rice production must increase dramatically. Increasing production of rice crops without changing technologies which affects water and nutrient utilization requires almost 300% more N alone than the present application rate in irrigated environments (Fischer 1998). This huge amount is an undesirable amount economically and environmentally. The productivity of rice production systems is governed by interactions between water availability and the nutritional status of the crop. That's why there is need of improved water and nutrient use efficiency of rice cropping systems for increasing productivity and profitability.

Breeding of improved varieties for tolerance to abiotic stress must be combined with management approaches which will be very useful to improve water and nutrient use efficiency. For rice systems, there is need of complete understanding of how soil nutrient supply is linked to cropping intensity, what pattern of nutrient supply is required to achieve the high yield levels needed to meet the needs of rice consumers in the next century, and how to improve agreement of nutrient supply and crop demand through management. To achieve higher productivity, improvement of varieties for efficient nutrient use can complement agronomic approaches. The

potential gains in improved nutrient acquisition and/or improved physiological efficiency are not clear for all nutrients, and these need to be estimated for better yield and genetically superior varieties. Improved screening techniques related to specific mechanisms of nutrient efficiency will facilitate genetic improvement.

Rice is a major consumer of agricultural inputs in the world, including water. Quality and quantity of available water is major concern for determining rice production. In the era of climate change, water availability is crucial. Serious concern has been raised regarding the depletion of surface water resources, reducing levels of groundwater, and lowering of water table due to rice cultivation in world.

Along with water, fertility status and nutrient-supplying capacity of soil in rice-growing area are very crucial factors. Rice crop accounts for 37% of the nitrogen and phosphate fertilizers' consumption (FAI 2013), besides 22% of the pesticides used in India (Krishna et al. 2002). The depletion of soil fertility and zinc (Zn) deficiency are major problems in rice–wheat cropping system (Timsina and Connor 2001). Among the nutrients, N is universally deficient in soil, and recovery of applied N is usually less than 50% (Fageria and Baligar 2005), while phosphorus (P) is noticed as a nonrenewable resource (Cordell et al. 2009) with a low availability due to slow diffusion and high fixation in soils (Shen et al. 2011). An increase in the agronomic use efficiency of N and P by microbial inoculation treatments over recommended dose of nitrogen makes them effective and profitable inputs for saving both water and nutrients and increasing water and nutrient use efficiency.

4.1 Changing Crop Established Methods

The major establishment methods of rice cultivation, puddled transplanted rice (PTR), aerobic rice system (ARS) (Prasad 2011), and system of rice intensification (SRI), differ in their water requirements (Uphoff and Randriamiharisoa 2003). SRI and PTR are found at par in yielding capacity (Singh 2013) and superior over ARS (Ram et al. 2006; Geethalakshmi et al. 2011; Singh 2013). Zhao et al. (2009) also reported higher irrigation and total WUE in SRI over PTR. The superiority of ARS in improving water productivity over PTR was discussed by Singh (2013).

In terms of water savings due to crop establishment methods, the lower depth of irrigation water application in SRI than PTR during the initial growth period was responsible for the saving of water in SRI (Zhao et al. 2009; Gopalakrishnan et al. 2014; Shahane et al. 2019). In ARS, the absence of nursery, no puddling, and maintaining arable soil result into saving of more water than both PTR and SRI. A comparative study is needed for assessing their potential in terms of water savings. In the era of climate change, water availability is critical (Mall et al. 2006; Madhusoodhanan et al. 2016), and with growing population-driven demand for rice (Kumar et al. 2009; Bhattacharyya et al. 2015), evaluating different cultivation methods for their influence on rice yield and water-saving potential is an important agenda.

4.2 Application of Microbial Inoculations

There are certain bacteria which contain special properties which are beneficent for plants. These bacteria are present in soil, and they affect the crops by fighting against harmful bacteria, and they are also the source of providing nutrition to the crops. Some bacteria like rhizobia and agro-bacteria are used to release seed inoculants and useful for the plants. The bacteria like *Azoarcus* are of much importance for the plants that it fixes the endophyte of the grasses. This type of bacteria is mostly helpful in rice crop, and they are very much environment friendly. When the seed is sown in the soil, bacteria play an important role in its germination. The bacteria grow in the seed and in return get food from it. Bacteria increase the fertility of the soil and provide such nutrients to the soil which are useful for the plant growth. They also help in softening the food in the seed, and this is the reason plants come out of the seeds. Though it is not certain what role bacteria play when the plants grow, they are of much importance in the early stages of plant development. Certain pesticides are developed using bacteria which give benefit to the crops (Satybhan et al. 2017).

In a developing country like India where more than 80% farmers belong to small and marginal class and most of them cannot manage to pay for expensive chemical fertilizers, the bio-fertilizers like blue green algae (BGA), *Azolla*, *Rhizobium*, *Azotobacter*, *Azospirillum*, *Acetobacter*, and phosphate solubilizing bacteria (Singh et al. 2017) can make the organic rice production system more worthwhile and decrease the ecological risks due to synthetic fertilizers. Among these, *Azolla* decomposes speedily, thus immediately providing the nitrogen to rice (Raja et al. 2012); and an average yield increase up to 1.4–1.5 t ha^{-1} could be achieved by effective *Azolla* inoculation (Mian 2002; Ciss and Vlek 2003). Similarly, *Herbaspirillum* is an endophytic diazotroph, which colonizes in rice roots (Baldani et al. 1986), and can fix 31–54% of total rice plant nitrogen requirement under gnotobiotic conditions (Baldani et al. 2000). Also, *Burkholderia* species, e.g., *Burkholderia kururiensis*, *Burkholderia tuberum*, and *Burkholderia phynatum*, hold potential of fixing N_2 (Vandamme et al. 2002), and its inoculation can increase grain yield in the range of 0.5–0.8 t ha^{-1} (13–22% increase). Lately, a *Rhizobium* strain has been confirmed to infect rice roots, traveling upward to stem and growing leaves and advancing its growth (Chi et al. 2005). The role of BGA, *Azolla*, and other bio-fertilizers in rice production is described hereunder:

4.2.1 The Blue Green Algae or Cyanobacteria

Algae are the heterogeneous assemblage of plants that includes prokaryotes and eukaryotic organisms. They are the pioneer colonizers both in hydrosphere and xerosphere and occupy the base of the tropic pyramid. These organisms have been found to synthesize 0.8×10^{11} tonnes of organic matter, constituting about 40% of the total organic matter synthesized annually on this planet. Blue green algae/cyanobacteria constitute the largest, most diverse, and widely distributed group of prokaryotic microscopic organisms that perform oxygenic photosynthesis. These are also known as myxophyceae, cyanophyceae, and cyanobacteria. These are ubiquitous in distribution, more common in tropics, and are able to withstand extremes of

temperature and drought. Organisms with ability to withstand adverse ecological conditions, capacity to thrive well in hostile environments, and response to the onset of dry conditions by entering into dormant resistant state have been distinguished as pioneers of plant succession. Nitrogen fixation is carried out in specialized cells known as heterocyst, which have thick walls and, hence, physically prevent the entry of oxygen and provide necessary anaerobic conditions for the activity of the enzyme nitrogenase.

The basic significance of the ecological observations on the abundance of BGA in Indian rice field soils became apparent when it was recognized that heterocystous forms can fix atmospheric nitrogen that is made available to the plants during life cycle and after its death by decomposition of cells which became available to the subsequent crops. Once nitrogen is fixed, many organisms are known to incorporate ammonia into amino acids by the enzyme GDH that is either absent or present in low amounts in these organisms. Under the conditions of ammonia limitations, GS–GOGAT is the major pathway for ammonia assimilation.

4.2.2 Use of BGA Bio-fertilizer for Rice Crop

De and Fritsch (1939) were the first to suggest a positive role of the blue greens in the sustenance of the nitrogen status of rice fields of our country. Since then lot of information has been generated in tropics regarding improvement in the fertility status of rice soils to sustain rice yields by utilizing diazotrophic BGA as biological input. Singh (1939, 1942) in Uttar Pradesh attributed the enhanced nitrogen contributions to the nitrogen fixing capacity of BGA, and the nitrogen contribution was calculated as 48 kg N/ha/crop. The All India Coordinated Project on the utilization of BGA in rice fields throughout the country revealed that the supplementation of chemical fertilizer input with blue greens could conserve up to 30% of the commercial chemical fertilizer. Multi-location trials conducted under varying agro-climatic conditions using different rice varieties indicated that the algal inoculation could save 30 kg N ha^{-1} that, however, depends upon the agro-ecological conditions. Venkataraman (1980) suggested the use of local or native strains for an efficient utilization and better crop response. BGA are also considered to minimize the occurrence of weeds in the rice fields (Subramanyan 1972) besides producing growth-promoting substances. Reclamation of the alkaline soils in Uttar Pradesh was demonstrated by Singh (1961) by introducing blue greens along with rice and sugarcane crops. These were reported to reduce the pH of soil and improve upon exchangeable calcium and water holding capacity. Under these situations, Na^+ ions are immobilized, and the sodium clay is converted to calcium type (Kaushik 1989). Rice fields in Kerala having acidic soils with low pH support the growth of several BGA (Anand and Shantha 1986). Many cyanobacteria exhibit a wide range of pesticide tolerance (Pabbi and Vaishya 1992).

4.2.3 Field Application Technique for BGA

The suggested method of application of the algal inoculum is broadcasting on standing water, about 3–4 days after transplantation. After the application of algal inoculum, the field should be kept water logged for about a week's time. Formation

of the algal inoculum can be detected within a week of inoculation in the form of floating algal mats, supplementary conspicuously seen in the afternoon.

BGA inoculum should be used to obtain 10–15% increase in crop yield. In organic rice cultivation, recommended pest control measures and other management practices do not normally interfere with the establishment and activity of the BGA in the field. Heavily fertilized rice fields generally show profuse growth of green algae that act as weeds and also reduce tillering in the rice plants. These can be differentiated from the BGA by the grass green color and fibrous nature. The green algae turn dark violet when treated with iodine, but the BGA remain unaffected. The green algae can be removed manually and buried in a pit and if their growth is intense. BGA bio-fertilizer should be used in every rice crop as a kind of insurance to the crop yield as well as to prevent the deterioration of soil physico-chemical properties.

4.2.4 *Azolla*

Azolla is an aquatic, heterosporous fern that occurs in a broad latitudinal range on five continents. This unique freshwater pteridophyte lives in symbiosis with the diazotrophic cyanobacterium *Anabaena azollae*. There are seven known species of *Azolla* in two extant sections, taxonomically separated by their secondary reproductive structures, including the number of float corpuscles (homologous to massulae) per megasporocarp and the form of glochidia that extend from the icro-sporic massulae. Several species are also distinguishable by the branching patterns or growth habits of their saprophytes.

4.2.4.1 Growth and Bio-fertilizer Potential of *Azolla*

The use of *Azolla* has been a part of rice cultivation in Vietnam and China for centuries and has been applied or tested more recently in other rice-growing countries. In addition to nitrogen supply, the benefits of *Azolla* as a green manure include provision of other mineral nutrients and organic matter to the soil. When established in rice fields, *Azolla* also reduces water evaporation and NH_3 volatilization. However, the realizable potential of *Azolla* as a green manure is restricted by climatic factors, water availability and quality, soil factors, mineral nutrition, and the need for labor-intensive management.

4.2.4.2 Field Application of *Azolla*

Field cultivation of *Azolla* entails a coordinated management to obtain rapid multiplication of inocula in rice fields or in adjacent bodies of water. By either means, the initial density of *Azolla*, when applied to the field, directly affects its successful establishment. Suboptimal densities encourage the presence of weeds. The amount of inoculum to be applied varies between 300–500 kg and 2–5 t ha^{-1} of fresh biomass. P amendments to the soil are generally required. Split applications are more efficient for rapid inoculum growth than are single basal applications. In Vietnam, field application of *Azolla* is combined with split applications farmyard manure. Ash is sometimes substituted for the chemical amendments. *Azolla* is first grown in small sections of the field until the surface of the water is covered; then half of the biomass

is transferred to new sections. The process is then repeated. The mat is intentionally fragmented to increase vegetative propagation. Alternatively, the harvested half of the *Azolla* biomass may be incorporated into the soil.

4.2.4.3 Agronomic Practices with *Azolla*

Three different management systems are possible with *Azolla*. One is to cultivate *Azolla* as a mono crop in the field and then incorporate it into the soil before transplanting rice. A variation of this method is to grow *Azolla* beforehand and to incorporate it as dried biomass. However, the mineralization rate is slower, and the nitrogen input is only half of that derived from live plants (Ito and Watanabe 1985). The second method is to grow the fern as a cover crop with rice to either decompose naturally or to be mixed into the soil as a top dressing. An inorganic nitrogen fertilizer may be added with the topdressing, or further intercropping of *Azolla* may be grown and incorporated into the field. Nitrogen is consequently available for the rice crop during growth and maturation. The third method, which makes nitrogen continually available to the crop, is to combine mono cropping and intercropping. The labor required for management of this complete utilization of *Azolla* is that much greater.

The decomposition rate of *Azolla* can be significant in its effect on rice plants, as extravagant green manuring may result in lodging and increased grazing or disease. Even the thickness of the *Azolla* mat must be considered, relative to its effect on the water temperature and C:N ratio. The timing and method of incorporating into the soil also affect *Azolla* effectiveness as green manure. The estimated fresh weight for a mat of *Azolla* is 10 t ha^{-1}, which is calculated to release 20–30 kg ha^{-1} nitrogen. Successive cropping of *Azolla* yields at least 100 kg ha^{-1} of nitrogen, which can produce a high-yielding rice crop.

4.2.5 *Azospirillum*

The genus *Azospirillum* colonizes in a variety of annual and perennial plants. It was observed that *Azospirillum* can enhance the growth of field crops, e.g., *Helianthus annuus*, carrot, oak, sugarbeet, tomato, pepper, cotton, wheat, and rice. The crop yield can increase from 5 to 30%. Inoculum *Azospirillum* can be produced and applied as in peat formulation through seed coating or seedling root dip treatment. The peat formulation can also be straightly applied in field applications.

4.2.6 Phosphorus Solubilizing Bacteria

Next to nitrogen, P is the vital nutrient for plants and microorganisms. This element is necessary for the nodulation by *Rhizobium* and even to nitrogen fixers, *Azolla* and BGA. The phospho-microorganisms mainly bacteria and fungi make available insoluble P to the plants. Direct application of rock phosphate is limited to acidic soil, while in other types of soil, the applied phosphate becomes insoluble within a short time. Monocalcium phosphates are converted to dicalcium phosphate which is slowly available to plants. The root fungus association or mycorrhiza has high potential in accumulating P in the plants. Combination of charcoal and soil is suitable substantial for these microorganisms in order to formulate marketable

inoculants. It is reported that microphos cultures increase yield up to 200–500 kg ha^{-1}.

4.2.7 Vesicular Arbuscular Mycorrhizae

The extent of mycorrhizal symbioses emphasizes the ancient evolutionary history and potential importance of fungal symbioses for plant production and physiology. The association between plants and their root-colonizing mycorrhizal fungi is a functional symbiosis in which the mycorrhizal fungus is obligatory or facultatively dependent on host photosynthates and energy. The plant-acquired carbon is dealt for numerous mycorrhizal reimbursements to the host plant. The mycological mycelium that spreads from the root exteriors into the soil atmosphere captures nutrients from soil solution. The tiny diameter of the fungal hyphae raises the surface area that the plants are able to exploit for their nutrient acquisition. Additional benefits from the mycorrhizal symbiosis include increased tolerance of heavy metal contamination or drought, as well as lesser susceptibility to root pathogens or herbivory. Mycorrhizal fungi may additionally improve soil quality by having an immediate influence on soil aggregation and therefore aeration and water dynamics. An interesting potential application for mycorrhizal fungi is their ability to permit plant access to nutrient sources typically unobtainable to the host plants. In summary, the various potential benefits of mycorrhizal symbiosis on plant performance and crop yield suggest that they have substantial applications in agriculture and in land reclamation or vegetation restoration. Possibly more than 80% of all land plants form mycorrhizal symbioses.

4.2.8 Plant Growth-Promoting Rhizobacteria (PGPR)

Various bacteria can promote plant growth and all such bacteria are termed PGPR. PGPR may be a generic abbreviation that indicates microorganisms, in some often-unknown way, can excite plant growth. PGPR are thought to expand plant by colonizing the root system and pre-empting the establishment of suppressing deleterious rhizosphere microorganisms on the roots. The PGPR improved potato growth and yield in short- but not long-rotation soils, primarily by suppressing cyanide producing deleterious rhizosphere microorganisms. Large populations of bacteria established on planting material and roots become a partial sink for nutrients in the rhizosphere, thus reducing the amount of C and N available to stimulate spores of fungal pathogens or for subsequent colonization of the root.

PGPR belong to several genera, e.g., *Azotobacter*, *Agrobacterium*, *Alcaligenes*, *Actinoplanes*, *Amorphosporangium*, *Arthrobacter*, *Erwinia*, *Bacillus*, *Cellulomonas*, *Rhizobium*, *Enterobacter*, *Flavobacterium*, *Pseudomonas*, *Bradyrhizobium*, *Streptomyces*, and *Xanthomonas*. These bacteria vary in their mechanism of plant growth promotion but generally influence growth via P solubilization, nutrient uptake enhancement, or plant growth hormone production. Rhizobacterium which has its place to the genus *Achromobacter* might increase the number and length of root hair in oilseed rape (*Brassica napus*). *Bacillus* spp. are appealing candidates for biocontrol because their endospores are tolerant to heat and desiccation. Seed treatment with *B. subtilis* increased yield of carrot by 48%, oats by

33%, and groundnut up to 37%, and it has been marketed as a treatment for groundnut in the USA. Currently *Pseudomonas* strains also suppress major plant pathogens.

4.2.9 Liquid Bio-fertilizer Formulations

The Division of Microbiology, ICAR-IARI, New Delhi, has developed liquid formulations of *Azotobacter*, potash solubilizing bacteria, and Zn solubilizing bacteria bio-fertilizers. These formulations have enhanced shelf life of more than a year and carry a high microbial load of 10^{10}–10^{12} cells/ml. They can be stored and are easy to deliver in the field at the most appropriate time. They can be used for the seed treatment, root dip for the seedlings in transplanted crops, and soil treatments. Inoculation with this single product can help to augment 15–20 kg N, 15–20 kg P_2O_5, and 5–10 kg K ha^{-1}.

4.3 Application Methods of Bio-fertilizers

4.3.1 Seed Treatment

One packet of carrier-based microbial inoculants (200 g) or one bottle of liquid-based inoculant (50 ml) culture is enough for treating seeds sown in 1 acre area. Make the solution of 100–150 g jaggery in water, and mix one packet of carrier-based microbial inoculants. Mix it thoroughly with the required seed for 1 acre so that each seed is coated with a thin film of culture. Spread the inoculated seed on clean and non-absorbent surface under the shade for drying at room temperature, and then seed should be sown on the same day. Liquid-based inoculant can be mixed with seed after dilution in required quantity of water.

4.3.2 Seedling Treatment

This method is recommended for crops like paddy, tobacco, vegetables, flowers, etc. where seedlings are transplanted. Prepare the suspension by mixing 200 g of microbial inoculant in a desired quantity of water. Uproot the seedlings required for 1 acre and make small bundles of seedlings. Dip the seedlings in the suspension for 15–20 min, and transplant the treated seedling immediately.

4.3.3 Soil Application

In case seed and seedling treatment is not possible, then 2–3 kg of the recommended bio-fertilizers are mixed in 200 kg of compost and kept overnight. This mixture is incorporated in the soil at the time of sowing or planting.

4.4 Application of Zn Fertilization

Along with water, fertility status and nutrient-supplying capacity of soil in rice growing area need due attention. The depletion of soil fertility and Zn deficiency are major problems in rice–wheat cropping system, particularly in India. Among the

nutrients, nitrogen is universally deficient in soil, and recovery of applied N is usually less than 50%, while P is receiving more attention as a nonrenewable resource with a low availability due to slow diffusion and high fixation in soils. Zn deficiency ranks fifth among the most important health risk factors in developing countries, and proper Zn management contributes about 18.4 million tonnes of grains (211.6 billion) for major food grain crops. Interactions between the root and the root microbiome influence plant growth as several microorganisms in the rhizosphere share a symbiotic or associative relationship with the plant. The use of microbial inoculation in the nutrient management of rice is known to elicit positive effects on growth, yield, and nutrient uptake.

Rice–wheat cropping system covers about 24 million hectares in China, India, Pakistan, Nepal, and Bangladesh, and Zn deficiency is widespread in rice–wheat belts of all these five countries. The current practice of applying zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) to soil is problematic because of the poor quality of the nutrients available in the market to the farmers. Zn-coated urea is therefore being manufactured to guarantee a good-quality Zn source. The relative efficiency of $ZnSO_4 \cdot 7H_2O$ and zinc oxide (ZnO) coated ureas in rice–wheat cropping system. The highest grain yield of rice–wheat cropping system was obtained with 2.0% coating of urea. $ZnSO_4 \cdot 7H_2O$ was also a better coating material than ZnO. Partial factor productivity, agronomic efficiency, apparent recovery, and physiological efficiency of applied Zn decreased as the level of Zn coating was increased (Shivay et al. 2008). Between microbes, the role of cyanobacteria and their consortium/biofilms with agriculturally beneficial bacteria in improving plant growth by N fixation and solubilizing soil P has been reported, and its promise is well established in conventional PTR system, besides recent reports on their promise in SRI, but not in aerobic rice. However, no information is available regarding the comparative efficacy of these inoculants in SRI and ARS, vis-à-vis PTR, in terms of improving yield and water savings.

Rice is very sensitive to low Zn supply in submerged paddy soils, and Zn deficiency is one of the major limiting factors in determining rice production. Its deficiency is prevalent worldwide, especially in the regions with high-pH calcareous soils (Adriano 2001; Cakmak 2002, 2008; Fageria and Baligar 2003; Norman et al. 2003; Prasad 2006; Alloway 2008; Prasad et al. 2014). Response of rice plant to Zn has been reported by several studies conducted in India (Srivastava et al. 2006; Shivay et al. 2007, 2010; Pooniya et al. 2012; Shivay and Prasad 2012), China (Tu and Feng 2000), and USA (Slaton et al. 2005).

Zn has been known as the fifth important risk factor for human health in developing Asian countries (IFA 2007). Therefore, efforts are underway to encourage Zn fertilization not only from the viewpoint of higher rice yield but also for increasing Zn concentration in grain and straw to improve human and animal Zn nutrition (WHO 2002). Furthermore, the increased Zn concentration in rice straw is of importance from the viewpoint of cattle nutrition, because in the developing countries of Asia, rice straw is the major feed for farm cattle (Shivay et al. 2008). Therefore, application of Zn as $ZnSO4 \cdot 7H_2O$ is generally recommended for rice and most other crops in India. Other Zn fertilizers include ZnO and Zn–

ethylenediaminetetraacetic acid (EDTA); the latter supplies substantial amount of Zn to the plants without interacting with soil components (Karak et al. 2005), because the central metal ion Zn^{2+} is surrounded by chelate ligands (Mortvedt 1979). Recent studies have shown that the effectiveness of Zn fertilizers in correcting Zn deficiency depends on the solubility of Zn fertilizers in water (Gangloff et al. 2002, 2006; Slaton et al. 2005; Shaver et al. 2007; Shivay et al. 2014, 2015a, b, 2016). The primary soil factors controlling the potential bioavailability of metals are soil pH, the accessibility and character of sorption sites on soil surfaces, the content of iron and aluminum oxyhydroxides, soil organic matter, and clay minerals (Gangloff et al. 2002). Some studies have indicated that organic sources are more effective fertilizers than inorganic sources. Their effectiveness depends on the rate of their disappearance from the soil solution, which is related to their stability (Alloway 2008). However, information on the relative efficiency of different Zn sources in the presence of large amounts of fresh organic residues from green manures is limited.

Almost half of the cultivated soils in the world are deficient in available Zn (Sillanpaa 1982). Low availability of Zn in alkaline soil is a long-lasting stress in cereal production in Turkey, India, Pakistan, and China (Singh et al. 2003; Cakmak 2004; Bell and Dell 2008). The reasons for spurt in Zn deficiency in Asian countries include introduction of high-yielding varieties of rice and wheat, application of high rates of high-analysis fertilizers such as urea and diammonium phosphate, removal of both grain and straw from the field at harvest, reduced to almost nil application of organic manures, and development and adoption of two to three crops a year intensive crop rotations, such as rice–wheat (Prasad 2005), resulting in mining of native soil nutrients. Zn application in crops has received additional consideration due to extensive Zn malnutrition specifically in developing countries (Gibson 2012; Graham et al. 2012) and the efforts being made for Zn bio-fortification of cereals, grains with Zn fertilization (Prasad et al. 2014).

The overall range of total Zn in soils is 10–300 mg kg^{-1} soil (Swaine 1955; White 1993) with average value of 50 mg kg^{-1} soil (Vinogradov 1959). Soils produced from basic rocks, such as basalt, is more affluent in Zn than those formed from acidic rocks, such as granite and gneisses (Vinogradov 1959). Further whole Zn is normally higher in heavier clayey soils than in lighter sandy soils (Frank et al. 1976). Zn in soils exists in unalike forms, such as, soluble, exchangeable, component of secondary minerals, organic matter connected, coprecipitated as secondary minerals, associated with sesquioxides and as a basic part of primary minerals (Shuman 1991). Hazra et al. (1987) conveyed that too much 84% of total Zn in soils arises as organizationally lattice bound, about 13% as sesquioxide bound, 1.6% as organically complexed, and about 1% as exchangeable and water-soluble forms. Only a minor portion of total Zn becomes available to crop plants. Soil extractants used for influential plant-available Zn contain dilute acids, such as 0.1 M HCl or a mixture of 0.0124 M H_2SO_4 + 0.05 M HCl (known as Mehlich I), or chelating agents, such as EDTA + $(NH_4)^2CO_3$ (Trierweiler and Lindsay 1969), 1 M ammonium acetate +0.02 M EDTA of pH 4.65 used in a global study by FAO (Sillanpaa 1982), revised Olsen's extractant (0.01 M EDTA + 0.5 M $NaHCO_3$ + 0.1 g L^{-1}Superfloc

126, pH 8.6) used by International Soil Fertility Evaluation and Improvement Project of North Carolina State University for use in Latin America (Hunter 1975), and 0.005 M DTPA + 0.01 M $CaCl_2$ buffered at pH 7.3 by 0.01 M triethanolamine (Lessman and Ellis 1971; Lindsay and Norvell 1978).

In soils, pH is the leading aspect significant in the accessibility of Zn. In alkaline and calcareous soils, Zn gets adsorbed or precipitated on hydroxide (especially those of iron) and carbonate surfaces. Zn also gets adsorbed or precipitated on negative charges of phosphates. On the other hand, in highly Cu-contaminated soils, Zn can get released in the soil solution. The interaction between Zn and other plant nutrients may not be a serious problem in cultivated soils. In plants, however, the interaction between Zn and other plant nutrients does exist, and both positive and negative interactions are reported. Nitrogen and potassium interact positively with Zn and rise its absorption and translocation in plants. P, calcium, iron, and copper react negatively with Zn and reduce its absorption by roots or/and its translocation to shoot in plants. As regards sulfur, both positive and negative interactions are conveyed. On the other hand, Zn application reduces boron uptake by plants, and Zn fertilization is recommended for alleviation of B toxicity in boron-rich soils.

5 Optimizing Nitrogen Options for Improving Water and NUE of Rice Under Different Water Regimes

Based on water regimes, soils, and climatic conditions, rice production systems are categorized into six different categories (Prasad et al. 2012), viz., irrigated (wet season), irrigated (dry season), rainfed upland, rainfed lowland, deep water, and coastal lowland production systems. Among them the irrigated rice production system covers about 44%, while rainfed production system covers about 45% of the global rice-producing area.

In order to face the challenge of booming demand for food and to fulfill the needs of an increasing population, the production of rice must be enhanced up to 70% by 2050 (Godfray et al. 2010). To achieve potential rice yields, modern cultivars of rice require a large number of fertilizers and other inputs. Among all fertilizers, N is the most essential for plant development, growth, and grain quality (Mae 1997; Kichey et al. 2007). However, in developed economies, N use efficiency is very low and estimated to be approximately 33% of the applied N source (Raun and Johnson 1999).

Water is crucial for the development and growth of rice crops. Substantial amount of inefficient water losses by evaporation, percolation, and seepage takes place in continuous flooding method of rice growing (Alberto et al. 2011; Shao et al. 2015). Nearly 80% of freshwater resources around the world is being consumed for irrigation purpose to produce rice in Asia, which accounts about 90% of world rice production (Bouman and Tuong 2001; Khush 2005). Because of climate change, rapid urbanization, and industrialization, water for agricultural utilization turned out to be progressively scarce (Bates et al. 2008; Yan et al. 2015). About 15 ± 20 million hectares area of irrigated rice in Asia will see water shortage by the end of 2025

(Tuong and Bouman 2003). To handle the increasing demand for food for the growing population, agriculturists are envisaging a challenge to deliver more per unit area rice with limited water availability. Approaches for efficient management of water for agricultural use involve conservation of water (both in situ and ex situ), integrated water use, better allocation of water, and enhancing WUE by various crops. In situ conservation of water can be achieved by reducing runoff loss and increasing infiltration of water and reduction of water loss through seepage and direct evaporation from the soil. Ex situ conservation of water can be accomplished by harvesting excess water in storage ponds for its reuse for irrigation. Efficient utilization of water resources can be achieved by the integrated use of water from different sources, viz., irrigation by profile-stored rainwater and conjunctive use of surface water and groundwater, good- or poor-quality water, and recycled (waste) water for irrigation. Supplemental irrigation for growing crops is an integrated use of rainwater stored in the profile and from other resources. Optimal allocation of available water among the competing crops and the right timing of application should be decided under adequate and limited water supply situation to maximize economic returns from available water. WUE by crops can be improved by selection of crops and cropping systems based on water availability and increasing seasonal ET. Control of ET is possible by selection of irrigation method, irrigation scheduling, tillage, mulching, and fertilization.

Efficient N fertilizer management can simply be defined as reduction in various losses and maximizing the amount of nutrient made available for crop (Ladha and Reddy 2003). The main challenge for breeders is to minimize the applied amount of N fertilizer to the field without affecting yield, and also in selecting the cultivars that metabolize N more effectively. Over-utilization of N causes lodging of plants, bringing a decrease in quality and quantity of rice yield (Cassman and Harwood 1995; Cu et al. 1996). Excessive use of N may induce the acidity of the soil (Guo et al. 2010) and water contamination (Diaz and Rosenberg 2008) and encourage nitrous oxide (N_2O) emission (Smil 1999).

Approaches used for nitrogen management of crops should be focused on two core principles: (1) it enhances beneficial use of externally applied fertilizer nitrogen as well as inherent soil N during the growing season itself, and (2) it conserves soil nitrogen by reducing the amount of N losses through various strategies and ensures higher beneficial use of this conserved N by the subsequent crops of the cropping system (Balasubramanian et al. 2002).

Various strategies based on above discussed approach for improving NUE are as follows:

Site-Specific Nitrogen Management (SSNM)

The rule of thumb of this concept is to establish an optimum synchronization between supply and demand of N for plant growth (Giller et al. 2004). Based on when and what type of decisions are made, SSNM can be categorized into two groups, A) prescriptive SSNM and (2) corrective SSNM. In prescriptive SSNM approach of N management, the amount and its application time are analyzed prior to sowing based on N-supplying power of the soil, expected crop N requirement for

assumed yield target, expected N efficiency of fertilizer products in use, etc. Opposite to this, corrective nitrogen management strategy involves use of diagnostic tools (qualitative measurement) to assess nitrogen status of standing crop. The interpretation of these recorded data serves the basis for decisions about timing and quantity of N applications (Schroeder et al. 2000). Chlorophyll meters, Nutrient Expert, and leaf color charts are promising and becoming popular in recent years for corrective N management in cereals (Yadav et al. 2017), especially rice.

Integrated Nitrogen Management (INM)
INM involves optimum use of all available N sources, i.e., crop residues, organic manure, biological N fixation, as well as chemical fertilizer and their complementary interactions to increase N recovery (Olesen et al. 2004).

Enhanced N Use Efficiency Fertilizers
These are fertilizer products that can improve N use efficiency by reducing various losses associated with production system and by enhancing their beneficial use in plants. These fertilizers are based on two concepts either by slowing the release rate of N or interfering with N transformation processes and reducing its losses. Slow-/controlled-release N fertilizers and nitrogen inhibitors are two important classes of fertilizers that lie within this category. Neem-coated urea is widely used and found to be slow-release N fertilizer in India. $NH4^+$ ion can be adsorbed on soil colloids and retained for a longer period which provides an opportunity for higher NUE by minimizing leaching and denitrification losses of applied N. In rice crop, dicyandiamide which is commercially available is used as nitrification inhibitor (Bharti et al. 2000).

Improved Method of N Application
There are various methods of N application, viz., deep placement and use of super granules and foliar spray of N fertilizer, which can enhance the recovery of applied N fertilizer. Broadcasting of nitrogen fertilizers is very common practice, but it leads to large N losses through the process of ammonia volatilization, which results in lower nitrogen recovery (McBratney et al. 2003). Use of modified form of N fertilizer such as urea super granules (USG) and deep placement of urea-based fertilizers has been reported to enhance NUE. Balasubramanian et al. (2002) reported, from large-scale demonstration in Australia, that recovery efficiency of N was 37% for broadcasting and 49% for deep placement of USG in rice; hence they emphasized that deep placement of N fertilizers can improve nitrogen recovery. Placement of urea with mud balls in the reduced zone of transplanted puddled rice field also improves N recovery and resulted into better crop output (Schmidt et al. 2002). Further, foliar feeding of nitrogen through urea spray in specific concentration can also improve NUE as it reduces different losses, i.e., runoff, volatilization, immobilization, and denitrification prior to being absorbed by the plant (Balasubramanian et al. 2002).

Adoption of Resource Conservation Practices
Adoption of various resource conservation methods, such as conservation agriculture, residue management, green manuring, and proper crop rotations, is found to be very promising for achieving better NUE.

Enhancement of NUE Through Genetic Improvement
Improvement in the crops genetics by introducing various quality traits responsible for effective N utilization may also enhance NUE.

Precision Farming
Precision farming is an information technology-based farm input management system which aims at the use of technologies and principles to identify, analyze, and manage spatial and temporal variability associated with all aspects of agricultural production in fields for getting maximum profitability and sustainability, enhancing crop performance, protecting land resources, and maintaining or improving the environment quality (McBratney et al. 2003). Measurement of variability in the field with respect to N and application of right amount of N at the right time by the use of techniques like variable rate applicator, remote sensing, geographic information systems, and global positioning systems technology may act as important information tools for the farmers to improve NUE under specific conditions of each field (Yadav et al. 2017).

Because of the significance of nitrogen as a major nutrient for rice crop to attain high grain yield, it is very important to determine the ideal amount and timing of N application for each rice cultivars and also the impact on agronomic parameters, for example, moisture content, plant height, lodging, and other parameters (Shrawat et al. 2008). Therefore, many scientists have identified different fertilization scheduling techniques through their experiments to achieve the maximum N use efficiency in rice fields (Deng et al. 2014). As per recommendation of scientists, nearly each farmer supplies N (Peng et al. 2011) in one single split or up to four splits during crop growth critical stages neglecting crop N demand and temporal changes to get high yields (Jing et al. 2007). Furthermore, a basal dose application of N on transplantation day or day before transplantation has been followed (Jing et al. 2007). The N loss is likely to be at the basal application as rice seedlings take 7 days in recovering from transplanting shock and after developing root, and rice N requirement is minimized during that period (Singh et al. 2002; Fan et al. 2009). However, to increase the rice yield, fertilizer application must match the indigenous N supply (Jing et al. 2007). N splits with basal and panicle initiation can increase yield (Russo 1996; Sathiya and Ramesh 2009). As per results of Prasad and Mailapalli (2018), four splits of N application (basal, tillering, panicle initiation, and heading) limit the NO_3^- leaching loss from rice fields. The adjusted splits could result in a reduction of N fertilizer input (Jeong et al. 2014), and splitting N can increase spikelet's per panicle, 1000-grain weight, ripened grain percentage, and N uptake (Pan et al. 2017).

Continuous flooding (CF) method provides a favorable water and nutrient supply under anaerobic conditions. However, the conventional system of irrigation

consumes a large amount of water (Nguyen et al. 2009). Several water-saving irrigation (WSI) technologies to reduce water use, to increase water use efficiency, and to maintain or increase production for rice-based systems have been developed (Li 2006). One of the most commonly practiced WSI techniques is alternate wetting and drying (AWD) irrigation (Belder et al. 2004). In AWD, supply of irrigation water to the field is done depending on the weather condition or until some fine cracks appear on the soil surface. Hameed et al. (2019) suggested from their experiments that splitting the N fertilizer resulted in high N use efficiencies at both AWD and continuous flooded (CF) conditions. Same reports were also suggested, for flooding irrigation in reference (Cabangon et al. 2004; Jing et al. 2007), that N use efficiency can be enhanced by increasing the number of splits and late applications. Ten-Berge et al. (1997) reported that small doses of frequent fertilizer application will result into better rice yield. Hameed et al. (2019) also reported that AWD is better than CF in terms of nitrogen utilizing efficiency. They explained that in AWD the high nitrogen uptake might be the reason of low nitrogen leaching which resulted in high N uptake and yield with high nitrogen efficiencies compared to CF. Re-watering in AWD can enhance the photosynthesis process in later stages, increases carbon remobilization from vegetative tissues to grains, and increases root biomass associated with high yields and nitrogen uptake (Wang et al. 2016).

Sun et al. (2017) reported through their experiment that there were presence of interacting effects of irrigation regime and N application strategies on grain yield and grain-filling characteristics as well. Researchers have reported a coupling effect between water and N on yield and N use in rice (Sun et al. 2016; Wang et al. 2016; Pan et al. 2017). Irrigation water reduction and high-efficiency fertilization in paddy fields have been the focus of recent researches for the theory and technique of high-yield and high-quality rice cultivation (Yang et al. 2003; Cao et al. 2017). Water and fertilizer are two main factors that mutually influence and constrain each other during rice growth and development (Wang et al. 2016). Some studies (Haefele et al. 2008; Wang et al. 2016) indicated that there was a significant interaction between N application and water management on grain yield in rice. Sun et al. (2017) showed that there were obvious interacting effects of irrigation regime and N application strategies on grain yield; however, the suitable N application patterns varied under different water regimes. Combined with the grain yield and grain-filling characteristics, Sun et al. (2017) concluded that a ratio of postponed N application to the total N application at 40–60% and panicle N application as topdressing at the emergence of the fourth and second leaves to the third and first leaves from the top were suitable under submerged irrigation conditions. The ratio of postponed N application to the total N application at 40% and panicle N application at the emergence of the fourth and second leaves from the top were the optimum water and N coupling strategy under alternate irrigation conditions. The ratio of postponed N application to the total N application at 20–40% and panicle N application as topdressing at the emergence of the fifth and third leaves from the top were suitable under dry cultivation conditions.

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Rice Breeding and Genomics Approaches for Improving Water and Nitrogen Use Efficiency

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Abstract

Rice is the most widely grown and consumed cereal across the world, where Asian countries are the topmost with 80%, since it meets half of the worldwide dietary. Rice is an extensive freshwater user through conventional puddled cultivation. However, as a result of changing climate, water scarcity is increasing, threatening rice cultivation. Therefore, it is imperative for genetic improvement of rice cultivars suitable to grow under water deficit conditions. While the significance of root traits in water uptake is unambiguously putative which is significantly correlated with higher water use efficiency. Besides water, plants need balanced mineral nutrients in each stage of its development to attain maximum yield. Among the essential nutrients, nitrogen (N) is one of the most important; with its deficiency, plants have limited growth, yield, and quality. Plants obtain N from the soil solution in two forms: $NO3^-$ and $NH4^+$. Since

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plants mainly uptake N as $NO3^-$ form root-specific transporters, but 1/3 of the applied N is leaching as $NO3^-$. However, earlier findings found that N use efficiency (NUE) may be enhanced through genetic improvement. There are two-gene (*NRT1* and *NRT2*) families of nitrate transporters encoding $NO3^-$ uptake proteins. Among them, *NRT2* gene encodes the high-affinity transporters under low nitrate concentration, while *NRT1* gene facilitates the root low-affinity transporters. In the chapter, we discussed various techniques/strategies in improving NUE and WUE in rice through phenotypic architecture particularly root and shoot, genetic engineering and molecular strategies in a range of plant species aimed at enhancing uptake, translocation, and remobilization of N as a sustainable way to increase rice productivity and quality and also and to incorporate into rice improvement programs through modern breeding and molecular approaches.

Keywords

Rice · Nitrogen · Root architecture · Water · Breeding strategy · Molecular approach

1 Introduction

Rice (*Oryza* sp.), one of the earliest domesticated crops, has been cultivated from the beginning of agricultural practices by human intervention. It is one of the most widely grown and consumed crops over the planet. Worldwide, scientists are working relentlessly for the improvement of its production for centuries. Over time, the rice-growing environment specially belowground soil properties has been altered a lot with extensive cropping, man-made hazards, and most importantly gradual but significant changes in global climate. Cultivation of this important crop became challenging at stressful growing conditions. Nutrients deficiency of plants as well as biotic and abiotic stresses interferes the optimum growth and development of rice, instigating a significant reduction in its final harvest. New nutrients are added in the list of deficient items in soil over the world regularly, within which nitrogen (N) is one of the most important members that requires a vast amount to ensure normal growth and development of rice. Farmers are using tons of N fertilizer in every corner of the world, which at the same time leads to a loss of huge amount of fertilizer to the environment at plant can only use a small portion of the applied N fertilizer. This also shifts the N balance leading to environmental pollution as well as increasing cultivation cost. On the other hand, water is very precious and becoming less available to be allocated for crop production, mostly for rice. So, introduction of new strategies or varieties is a must which will ensure the best use of per unit N and water to produce maximum yield. In recent days, nitrogen use efficiency (NUE) and water use efficiency (WUE) have been a major target of rice scientists. The objective of this chapter is to accumulate the information of various techniques/strategies in improving NUE and WUE in rice and to incorporate into rice improvement programs through modern breeding and molecular approaches.

2 Nitrogen Use Efficiency (NUE)

Nitrogen is a mineral nutrient essential for normal growth and development of plants that is required in the highest quantity (Taiz and Zeiger 2006). It is an element of nucleic acids, proteins, and photosynthetic metabolites, therefore crucial for rice growth and metabolic processes (Noor 2017; Ghoneim and Ebid 2015). The efficient use of N chemical fertilizers can be attained through cultural and agronomic practices. Nitrogen use efficiency is an important trait that has been studied for decades in different crops. The grain production or economic return from the per unit supply of N fertilizer simply explained the NUE. Several definitions were suggested by different researchers (Moll et al. 1982; Good et al. 2004; Fageria et al. 2008; Moose and Below 2009). NUE can be defined as the product of N uptake efficiency (NUpE) and N utilization efficiency (NUtE). An increase in NUE increases the yield, biomass, quality, and quantity of crops (Raun and Johnson 1999; Tilman et al. 2002; Masclaux-Daubresse et al. 2010). Nitrogen is generally applied as chemical fertilizer to the soil, whereas a small amount is added to some crops like grain legumes through fixation process. On the other hand, crop plants take N through the root system in the form of nitrate or ammonium which thereby used in different metabolic processes (Stitt et al. 2002). As a mobile element, N loss is greater than any other element from the soil. Moreover crop plants varied in their ability to uptake N for their metabolism. Despite the positive implications of N in production, most plants can only uptake 30–50% of the supplied N based on soil, environment, and plant population (Tilman et al. 2002). Volatilization, denitrification, and leaching are the major reasons for N losses from the soil. In the process, N pollutes the air and water (Wuebbles 2009; Ng et al. 2016; Shen et al. 2013). The secondary impact which is directly related to economic return is cost of production increases as plant doesn't uptake more than 50% of the N applied (Han et al. 2016a). A recent report indicated that in 24–39% growing regions, crop yields including rice had not improved and remain static (Ray et al. 2012), as compared to crop yields applied with fertilizer (Shen et al. 2013). So, utilization of N fertilizer in improving rice yields need to be optimized or NUE need to be improved. These could reduce environmental pollution as well as production cost.

A number of studies have been conducted in several decades to increase the NUE in different crops including rice and indicated that NUE can be improved by conventional breeding as well as molecular approaches through genetic engineering (McAllister et al. 2012). But later one has not been extensively studied in rice compared to traditional approaches that target NUE (Han et al. 2016a).

3 Water Use Efficiency (WUE)

Rice is one of the most important staple foods for mankind worldwide. Water is the most precious input for rice cultivation. Different parameters like specific leaf area, isotopic carbon discrimination, leaf chlorophyll level (SPAD value), and root traits, especially root length, density, and depth, have long been seen as critical traits in

order to improve water use efficiency and crop adaptation in nonoptimal environmental conditions. The WUE is simply defined as the amount of dry matter (or grain yield) produced per unit of water consumed. It can also be defined as the ratio of photosynthetic carbon assimilation to transpirational water loss. Generally, WUE is measured through its components or related characteristics. The size and activity of the root system determine the rate at which the shoot system can produce photosynthates. It is evident that improving yield under stress conditions will require a whole-plant approach. A key factor determining plant productivity under drought conditions is WUE, and it is mentioned as a strategy to improve crop performance under limited water conditions (Araus et al. 2002; Dharmappa et al. 2019). WUE is an important trait associated with drought resistance of crop plants (Mian et al. 1998), and crop yield under drought is related to water uptake, WUE, and harvest index. Genetic improvement of WUE could enhance the drought resistance of crops (Specht et al. 2001). Breeding rice varieties is one of the options to develop higher water use-efficient rice varieties. Quantitative trait locus (QTL) mapping allows for improved understanding of the genetic control and inheritance of WUE and can indicate which selection strategy should be adopted though QTLs can vary from population to population and are influenced by the growing environment. Therefore, identification of QTLs under near realistic field stress conditions will enhance pace of its use in molecular breeding and genetic engineering. Map-based cloning of major genes that control the QTLs involved in WUE and drought tolerance and validating their function in rice transgenic will be an important step toward genetic engineering and molecular breeding to enhance WUE in rice. Impa et al. (2005) described that WUE has the most intimate relationship with drought tolerance in rice. Agronomic parameters like photosynthetic rate, relative water content (RWC), and stomatal conductance have strong positive correlations with WUE, whereas transpiration rate expresses negative correlation with WUE under drought in rice varieties (Akram et al. 2013). Xu et al. (2009) detected seven QTLs for leaf WUE in a population of 98 BILs derived from a cross between temperate *japonica* and *Aus* rice at the seedling stage with carbon isotope discrimination as the criterion. The QTL with the largest additive effect was from *Aus* rice and was co-localized with QTLs for leaf length, tiller number, and N content.

4 Phenotypes to Be Targeted for NUE and WUE

4.1 Belowground Traits (Root)

Plant roots, the hidden half that often get less attention by the researchers, are the important parts of plants as nutrient and water uptake takes place through the root system. Besides, soil environment study, stress study, root physiological study, nutrient uptake mechanism, etc. mainly rely on it. NUE- and WUE-related traits are mostly root-related traits that include root morphology, root-shoot ratios, root vigor, root length density, root hair, root N transport, metabolism, etc. Most of the roots included in the study focused on the seedling stage in seedbed or pot culture

(Hund et al. 2009; Bai et al. 2013; Atkinson et al. 2015). Other researchers extrapolated that information in field condition (Manske et al. 2001; Wasson et al. 2014; Trachsel et al. 2011) as significant role of interaction between plant root and soil environment (Wojciechowski et al. 2009). There are also environmental studies regarding different crops based on uprooting process of plants which may involve excavations, removal of soils, rinsing, cleansing, etc. that lead to inaccurate phenotyping (Passioura 2006, 2010; Poorter et al. 2012). Another difficulty is that recording the root trait observations sometimes increases the error during counting of secondary/tertiary roots as well as few other traits. Though nowadays root imaging/scanning techniques are getting popular due to its less laborious processes (Metzner et al. 2015; Manschadi et al. 2006, 2010; Schneider et al. 2012), its accuracy is under question and depends on the appropriate software program utilization.

4.2 Aboveground Traits (Canopy)

Aboveground NUE and WUE traits are mainly canopy of the plants. Phenotypic traits of canopy including canopy biomass, canopy cover, greenness index, chlorophyll content, stomata associated traits, leaf-related traits (senescence, drying, blight, rolling, and others), etc. There are currently lots of options to record canopy-related traits. But high-throughput phenotyping of canopy traits is still challenging in crop improvement. Spectral reflectance-based technologies are available for recording such observations (Cormier et al. 2016). The normalized difference vegetation index (NDVI) is one based on light absorption by chlorophyll and other pigments (Araus et al. 2001). A number of spectral reflectance indices (SRI) have been developed to estimate different traits like canopy coverage (Aparicio et al. 2002), "stay-green" (Lopes and Reynolds 2012), chlorophyll content (Babar et al. 2006), grain yield (Gutierrez-Rodriguez et al. 2004; Gutierrez et al. 2010a, 2010b), crop biomass (Babar et al. 2006), and grain protein content (Apan et al. 2006; Freeman et al. 2007). Apart from this, sensor-based instrument has been also developed in recent days as chlorophyll fluorescence imaging to measure photosynthesis (Romer et al. 2011; Murchie and Lawson 2013) and infrared thermometry for canopy photosynthesis (Olivares-Villegas et al. 2007; Saint Pierre et al. 2010). Laser imaging detection and ranging (Lidar) is another instrument that could estimate accurately the plant height, canopy coverage, crop biomass, N content, etc. (Lefsky et al. 2002 ; Omasa et al. 2007; Hosoi and Omasa 2009; Eitel et al. 2014). Quite a few reviews on such technologies were published in recent years (Furbank and Tester 2011; White et al. 2012; Araus and Cairns 2014).

5 Underlying Biological and Molecular Processes for NUE

5.1 Targeting N Transporter

Nitrogen is present in the soil in the form of nitrate (NO_3^-) or ammonium (NH_4^+) in aerobic or flooded conditions, respectively. The uptake of both NO_3^- and NH_4^+ from the soil through root-specific transporters involves two physiological mechanisms. At low N concentrations (<250 μM), high-affinity transport system (HATS) by *Nitrate Transporter 2 (NRT2)* and *Ammonium Transporter 1 (AMT1)* were involved for NO_3^- and NH_4^+ uptake, respectively, while the low-affinity transport system (LATS) by *NPF (NRT1/PTR)* generally plays role under high N concentrations (>250 μM) (Dechorgnat et al. 2011; Leran et al. 2014).

In rice, three *OsAMT1* for high-affinity while seven members of *OsAMT2*, *OsAMT3*, and *OsAMT4* for low-affinity were characterized as a controlling factor for NH_4^+ transportation (Li et al. 2009). Expression of gene encoding transporter proteins may be at fixed organs of plants, while others may express differentially (Saiki et al. 2003; Kumar et al. 2003; Suenaga et al. 2003). In ammonium-preferring rice cultivar, *AMT* members could be more effective than nitrate-preferring one in the context of NUE (Ding et al. 2011). Rice mutants of *OsAMT1.1* transporter has already been utilized to increase NUE as the form of NH_4^+. It has a tendency to reduce growth in roots and shoots at high as well as low ammonium concentration (Li et al. 2015). On contrary, differential results of no significant reduction were reported where *OsAMT1.1* overexpressed in different rice varieties: Kaybonnet, Jarrah, and Taipei (Ranathunge et al. 2014; Kumar et al. 2006). The ammonium transporter *OsAMT2.1* were expressed under different N source, whereas *OsAMT3.1* expressed weakly (Suenaga et al. 2003). A number of other studies have been executed in relation to ammonium transport in rice involving *OsAMT* genes with limited success (Hoque et al. 2006; Ranathunge et al. 2014; Bao et al. 2015; Sonoda et al. 2003).

Although the uptake of NO_3^- is very less in rice compared to NH_4^+, its effective uptake could contribute to increase the NUE and grain harvest of rice (Wang et al. 2018). Rice root aerenchyma is involved in oxidizing ammonium to release oxygen at the root zone, resulting to NO_3^- formation which is then taken up by plants (Kirk and Kronzucker 2005). Nitrate transporter1/peptide transporter *(NRT1/PTR)* family genes are known for transporting several substrates including nitrate, amino acids, peptides, glucosinolates, IAA, ABA, etc. (Leran et al. 2014). This family regulates the allocation of NO_3^- in shoots from root in plants (Tang et al. 2012; Fu et al. 2015; Li et al. 2015; Han et al. 2016b; Fan et al. 2016). Gene families *NRT1* and *NRT2* regulate low- and high-affinity transporters under low nitrate conditions (Williams and Miller 2001). Although there are more than 80 genes identified for *NRT1* and *NRT2*, only few were characterized by the *NRT1* family (Cai et al. 2008; Inostroza-Blancheteau et al. 2017). The first identified low-affinity transporter *OsNPF8.9 (OsNRT1)* in rice is associated with N uptake through root epidermis and hairs (Lin et al. 2000), and its overexpression enhances the N concentration in rice plant (Fan et al. 2015). PTR gene *OsNPF4.1 (SP1)* expressed in phloem of panicle

controls panicle size (Li et al. 2009), while *OsNPF7.3 (OsPTR6)* involves in glutamine synthetase and N uptake (Fan et al. 2014). *OsNPF6.3 (OsNRT1.1A)* regulates the N utilization and shortened flowering process in rice (Wang et al. 2018), and *OsNRT1.1B* encompasses nitrate uptake and transport (Hu et al. 2015). Till date, four *NRT2* and two *NAR2* genes reported in rice are of high-affinity transporter group, in which *OsNRT2.3b* and *OsNRT2.4* independently, whereas *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3a* with the interaction of *OsNAR2.1* mediates the nitrate uptake (Araki and Hasegawa 2006; Cai et al. 2008; Feng et al. 2011; Yan et al. 2011). Transgenic rice having *OsNRT2.1* transporter containing *OsNAR2.1* promoter showed improved NUE and could produce higher yield than the wild one (Chen et al. 2016a, 2017). *OsNRT2.3a* expressed in root steles had a role in transporting nitrate from root to shoot (Tang et al. 2012), while *OsNRT2.3b* expressed in leaf involved in nitrate remobilization, photorespiration, and growth enhancement (Fan et al. 2016) and also increases NUE and yield in rice (Fan et al. 2017). On the contrary, rice mutant of *OsNPF2.2* associated with impaired growth and grain filling (Li et al. 2015) and *OsNPF2.4* associated with lessened nitrate transportation (Xia et al. 2015) with root aerenchyma released oxygen in rhizosphere.

5.2 Targeting N Assimilation

Nitrate (NO_3^-) and ammonium (NH_4^+) are major N resources for plant uptake. Once nitrate is incorporated in plant cells, it is first reduced to nitrite by a *nitrate reductase (NR)* in the cytosol (Meyer and Stitt 2001). Then nitrite is imported into the plastids and chloroplasts where it is reduced to ammonium by the *nitrite reductase (NiR)* enzyme. Ammonium derived from nitrate, or the one produced by photorespiration or amino acid recycling, is mostly assimilated in the plastids by the *GS/GOGAT (glutamate synthase)* cycle (Masclaux-Daubresse et al. 2010). GS is a very important enzyme for N assimilation and remobilization, and there are two isoforms of the enzyme: *GS1* that carries out primary ammonium assimilation in roots or re-assimilation of ammonium in leaves and *GS2* that carries out assimilation of ammonium in the chloroplast. In rice, there are three GS members; *OsGS1.1* and *OsGS1.2* are expressed in all organs and showed a reciprocal response to ammonium supply in the cell layers of the root surface. Depending on the electron donor specificity, there are two kinds of *GOGAT*, *ferredoxin-dependent (Fd-GOGAT)* and *NADH-dependent (NADH-GOGAT)* enzymes. In rice, one ferredoxin and two NADH-dependent enzymes exist (Tabuchi et al. 2007). Improvement of N assimilation efficiency is critical for efficient use of N (Xu et al. 2012). Carbon (C) assimilation is also involved in the process, and a lot of enzymes, so enzyme activity detection is crucial in developing rice variety having high NUE (Masclaux-Daubresse et al. 2010; Kurai et al. 2011; Thomsen et al. 2014). Quite a lot of studies have been designed and executed in improving NUE through the overexpression of N assimilation genes (Good et al. 2004; McAllister et al. 2012; Xu et al. 2012); but

the outcome is less consistent, while a majority of the transgenic plants need to be evaluated under field conditions.

In a study, overexpression of *OsGS1.1* and/or *OsGS1.2* reported the enhancement of GS activities but without a significant change in rice yield (Cai et al. 2009); while NUE improvement is reported by overexpression of *OsGS1.2* only under the growth chamber (Brauer et al. 2011). Mutation of *OsNADH-GOGAT2* (like *OsGS1.1*) resulted in reduced spikelet number, growth rate, and grain filling (Tabuchi et al. 2005; Tamura et al. 2011); and *OsNADH-GOGAT1* (like *OsGS1.2*) mutant is reported for impaired amino acids and ammonium ions and also for reduced tiller number (Tamura et al. 2010; Funayama et al. 2013; Yamaya and Kusano 2014). The differential results of increased grain weight in indica cultivar by the overexpression of gene *NADH-GOGAT* (Yamaya et al. 2002); while regulation of ammonium assimilation in seeds was reported with the *OsGS1.3* (Tabuchi et al. 2007).

5.3 Targeting N Remobilization

Remobilization is the process of moving N from old senescing plant parts to younger parts during vegetative phase or to the storage organs during the reproductive phase (Schiltz et al. 2005). Nitrogen remobilization is a complex metabolic process, but it is considered as one of the important steps in improving NUE in plants (Masclaux-Daubresse et al. 2010). In case of cereals, grains main source of N is found to be N remobilized from the vegetative parts. It accounts for 60–92% of the N accumulated in grain, and the remobilization rate depends upon the availability of N and remobilization efficiency. Germination and subsequent seedling survival are regulated by N in seeds. For efficient remobilization of leaf N, there should be tight coordination between remobilization and senescence-induced protein degradation as senescence leads to N translocation to the reproductive parts of the plants through phloem. In rice, phloem transfers 80% of the panicle N from senescing organs to panicle. In this process, GS and GOGAT enzymes regulate N remobilization in senescing organ and reutilization in reproductive organs (Tabuchi et al. 2007). *GS1.1* and *NADH-GOGAT1* have essential role in those processes (Tabuchi et al. 2007; Hayakawa et al. 1994). Delayed leaf senescence is important for grain yield as prolonged photosynthesis contributed to achieving higher yield but reduces N remobilization and subsequently protein content in grain (Masclaux-Daubresse et al. 2010). A number of genomics studies and QTL detection were accentuated in relation to GS involvement in N remobilization and reproductive efficiency in many crops (Hirel et al. 2001; Obara et al. 2004; Bernard and Habash 2009). In the case of rice, there are three *Gln1* genes detected which encode *GS1* (Bernard and Habash 2009). These Gln1 genes are found to be located at different tissues in plant with different isoforms and had different functional properties (Ishiyama et al. 2004). For this reason, attempts have been made to identify genes that can encode proteins during N remobilization and senescence (Gallais and Hirel 2004). Brugiere et al. (2000) suggested that cytosolic GS (*GS1*) reassimilates ammonium released from protein hydrolysis, which mediates *Gln* synthesis in phloem sap and controls N

remobilization in rice (Andrews et al. 2004). Rice mutants lacking *OsGS1.1* were reported for reduced growth and grain filling (Tabuchi et al. 2005). In another study, *OsGS1.1* gene found to be involved in glutamine generation and consequently in remobilization through the phloem (Obara et al. 2001, 2004). Studies revealed that *OsGS1.1* is essential for growth and yield of rice and *OsGS1.2* and *OsGS1.3* cannot become an alternative to *OsGS1.1* (Tabuchi et al. 2005, 2007).

5.4 Targeting Molecular Components

The transcription factors and the driving force behind the plant's phenotypic expression. Apart from the transporters and enzymes, many regulatory and transcription factor genes are involved in NUE regulation in plants that play important roles in nutrient uptake, redistribution, assimilation, and storage. NO_3^- is a signaling molecule in plant system, and genes like *AtNPF6.3/NRT1.1* and protein kinases like *AtCIPK8* and *AtCIPK23* involved in NO_3^- signaling (Ho et al. 2009; Hu et al. 2009), and the enhancement of N assimilation and plant growth in rice were observed by overexpression of this gene at low N concentrations (Kurai et al. 2011).

Hormone signaling and tissue differentiation and some other biological processes are mediated by *DOF* (*DNA-binding One Zinc Finger*) transcription (Noguero et al. 2013). In transgenic rice plants having *Zea mays Dof1* (*ZmDof1*), gene expression enhances the N and C assimilation in roots and photosynthesis rates (Kurai et al. 2011). Similar to this finding, another transgenic rice with an expression of maize *FERREDOXIN-NADP+ REDUCTASE* gene was found with better cob size and kernel weight along with more root growth (McAllister et al. 2012). A QTL *Gn1a* was involved in increasing yield in rice (Ashikari et al. 2005). An increase in uptake N responsiveness, as well as grain productivity, was recorded by the overexpression of transcription factor *Dof OsRDD1* in rice (Iwamoto and Tagiri 2016). A gene *DOF18* induces the ammonium transporter *AMT1*, *AMT2*, and *AMT3* in controlling ammonium uptake in the root tissue of rice (Wu et al. 2017).

Several studies reveal the crucial roles of G-protein pathways in rice development and N use. A major QTL *Dense and Erect Panicles 1* (*DEP1*) for panicle architecture found to have a function in increasing the panicle number and ultimately the yield (Huang et al. 2009a). The mutated allele *DEP1* reported to have an association with the transcript of transporter *OsAMT1.1* and N uptake in plants (Sun et al. 2014). *AtHY5* is a transcription factor involved in light regulation (Lee et al. 2007) and N uptake and assimilation (Jonassen et al. 2009; Huang et al. 2015; Chen et al. 2016b). In rice, higher GS activity cultivar was found to recycle NH3 and left less amount of NH3 to lose compared to low activity one (Kumagai et al. 2011).

6 Underlying Biological and Molecular Processes for WUE

6.1 Targeting Root Traits in Boosting Water Uptake

Plant roots play an important role in water and nutrients uptake, which is necessary for plant growth and development. They also act as storage organs and basic plant parts in the soil. Plants having different root characteristics and morphologies are able to increase water uptake efficiency and to adapt in different ecosystems by responding to its surrounding environments if they are affected by external environments (Bao et al. 2014; Robbins and Dinneny 2015). The plant root system is a primary target for developing new varieties or to continue normal growth under unfavorable environmental conditions. Breeding for superior root systems may be crucial for selecting water-efficient crops that can lead to efficient uptake compatible with high yields (Blum 2009; Kell 2011; Palta et al. 2011). In many plants, drought has a substantial impact on the development of the root system specially increasing primary root elongation and repressing lateral root branching. Lateral root formation is a determining factor of overall root system architecture and sum of the total root biomass, total root length, and root surface area. Lateral root density depends on both genetic and soil conditions. Greater nutrient and water uptake are positively related to lateral root density (Bao et al. 2014; Tian et al. 2014). In rice, the NAC family of transcription factors was characterized with regard to root architecture (Zheng et al. 2009). Redillas et al. (2012) assessed the overexpression of the TF OsNAC9 in transgenic rice under the control of a constitutive or root-specific promoter in both normal and drought conditions. In suboptimal water availability, transgenic rice lines showed a reduced lateral root density but exhibited higher grain yield. Seo and Park (2009) noted that MYB96 transcription factor, an *Arabidopsis* R2R3 type, controls lateral root development via ABA-auxin signaling crosstalk under drought stress conditions. The MYB96-overexpressing mutant having dwarfed growth and reduced lateral root formation performs an improved drought tolerance and a significantly elevated expression of the GH_3 genes. On the other hand, the MYB96-deficient knockout mutant had more lateral roots and was more susceptible to drought stress.

Under mild water deficit, the root growth usually maintains while shoot growth is suppressed. In roots, drought results in the reduction of meristematic activity, arresting root elongation. Root dry mass and length are good indicators of rice yield under drought stress (Fageria and Moreira 2011; Feng et al. 2012). Lines containing qDTY12.1 exhibited more lateral root and root hair formation and also found higher potential to increase grain yield under drought stress (Dixit et al. 2015). Rice genotypes that have deep, coarse roots with a high ability of branching and penetration and higher root-to-shoot ratio are considered as component traits of drought avoidance (Samson et al. 2002; Wang and Yamauchi 2006; Gowda et al. 2011). Coarse roots have direct roles in drought resistance because larger diameter roots are related to penetration ability (Nguyen et al. 1997; Clark et al. 2008) and branching, and they have greater xylem vessel radii and lower axial resistance to water flux (Yambao et al. 1992). The capacity for deep root growth and large xylem diameters in deep roots may improve root acquisition of water when ample water at

depth is available, while small xylem diameters in targeted seminal roots save soil water deep into the soil profile for use during crop maturation. Henry et al. (2012) suggested that lower xylem sap bleeding rates from roots, more stable hydraulic conductivity with variation in soil moisture, more responsiveness of root anatomy to drought, and greater levels of aquaporin expression are component traits for drought resistance in rice. Under drought stress, rice plants exhibited decrease suberization and compaction of sclerenchyma layer cells, but water retention was found to increase in the same condition. Another factor that influences rooting depth is gravitropism. Uga et al. (2013) examined the gravitropic response of rice roots having deeper rooting 1 (DRO1) quantitative trait loci. They found rice plants leads to more drought tolerance due to their deeper root system via steeper root angles and more robust seedling gravitropic responses.

The transpiration is considered as the only productive water outflow due to photosynthesis and leaf cooling at the field level though only a fraction of transpiration is actually beneficial and the remainder is wasteful. The role of transpiration in keeping the leaves cool is a potential source for breeders. As stomatal transpiration and nonstomatal transpiration decline, is it possible that leaf temperature will rise and inhibit production? One possible way of dealing with this issue is to enhance the heat tolerance of crops by non-transpirational means. Two such approaches are through the expression of genes for the heat-shock 101 (HS101) proteins (Queitsch et al. 2000) and ascorbate peroxidase (Shi et al. 2001). In addition to it, the ability of plants to cool their canopy temperature through transpiration needs efficient functioning of the root water uptake system. QTLs controlling root morphological traits in rice (Babu et al. 2003; Courtois et al. 2003; Zheng et al. 2003) have been identified by using molecular markers. Steele et al. (2002) have undertaken the introgression of QTLs for root traits in MAS breeding for enhanced drought tolerance and WUE. In rice, QTL have been mapped for root morphology, root distribution, and drought avoidance (Champoux et al. 1995; Price and Tomos 1997; Yadav et al. 1997; Ali et al. 2000; Courtois et al. 2000; Kamoshita et al. 2002); root penetration ability (Ray et al. 1996; Price et al. 2000; Zheng et al. 2000); osmotic adjustment and dehydration tolerance (Lilley et al. 1996; Zhang et al. 1999); stomatal conductance, leaf rolling, and heading date (Price et al. 1997); cell membrane stability (Tripathy et al. 2000); and ABA accumulation (Quarrie et al. 1997). The marker-assisted selection (MAS) and map-based cloning for root traits are sometimes difficult, since the contribution of a QTL to the variance is often quite small. Thus, several QTL related to root traits are needed to be pyramided to reconstruct the trait to an adequate extent for better performance at normal conditions or even under drought stress.

6.2 Targeting Stomatal Control Mechanism in Reducing Water Loss

Water loss from the plant to the atmosphere occurs through transpiration mediated by stomata and direct evaporation of water from the epidermal cell surface. Of these two, the majority of the water loss is occurring through the stomata. The number of

stomata in developing leaves can vary with different environmental factors like temperature, humidity, light, atmospheric carbon dioxide, and drought. Stomatal pores allow CO_2 influx for photosynthetic carbon fixation and water loss via transpiration to the atmosphere. Thus, the rate of transpiration and photosynthesis depends upon the plant's ability to regulate its stomatal pores. The amount of transpiration will reduce when crops will close the stomata, reducing absorption, sweating decreases, or a combination of all three levels in order to prevent water losses (Shekari 2000). Thus, higher intrinsic water use efficiency (WUE) can be associated with both reduced stomatal conductance and higher photosynthetic capacity.

To control stomatal responses, plants are utilizing the stress hormone, namely, abscisic acid (ABA), synthesized by root under receding soil water conditions or by leaf when the transpiration exceeds water uptake. QTLs for traits that minimize water loss through plants such as controlling stomatal regulation (Price et al. 1997), leaf ABA accumulation (Quarrie et al. 1997), and leaf rolling (Courtois et al. 2000) have been reported and tagged with molecular markers in rice. These QTLs and genes involved in minimization of water loss can be used to genetically modify the stomatal regulation and improve WUE. During drought conditions, plants synthesize and accumulate abscisic acid (ABA) in the guard cells to help activate closure of the stomata in order to reduce the amount of water lost (Lim et al. 2015). The reduction of water content reduces stomatal activity and cell growth. Hu and Xiong (2014) described that LOS5/ABA3, a major enzyme in the final stage of ABA biosynthesis, was overexpressed in transgenic rice and the grain filling and grain yield were enhanced under drought conditions. Rice zinc finger protein (dst mutant) showed improved drought and salt tolerance by reducing the stomatal density and increasing stomatal closure. Conversely, DST nonmutants act negatively on stomatal closure by modifying H_2O_2 homeostasis (Huang et al. 2009b). In rice, Ishimaru et al. (2001) studied the genetic relation between adaxial and abaxial stomatal density with quantitative trait loci (QTL) analysis on a population of backcrossed inbred lines of japonica variety, Nipponbare, and indica variety, Kasalath. Four QTLs controlling adaxial and abaxial stomatal frequencies were identified on chromosome 3; the QTL for adaxial density overlapped with the QTL for abaxial density, suggesting that the same locus may pleiotropically control stomatal density on both surfaces of the leaf. Laza et al. (2010) detected 4 QTLs controlling the size of stomata and 10 QTLs controlling stomatal density across growth stages and leaf surfaces, using 101 recombinant inbred lines (RILs) derived from a cross between the japonica IR69093-41-3-2-2 and the indica IR72. The contributions of the QTLs ranged from 9.7% to 14.3% for stomatal size and 9.3% to 15.2% for stomatal density.

Carbon isotope discrimination (CID) has been recognized as an indirect tool for selecting plants having higher WUE and yield potential. CID has an inverse correlation between transpiration efficiency (Dingkuhn et al. 1991; Scartazza et al. 1998; Cabuslay et al. 2002; Kondo et al. 2004) and WUE (Impa et al. 2005) at the leaf level in rice under drought stress. In general, water stress increases carbon isotope ratio ($\delta^{13}C$) and decreases CID values in rice (Kondo et al. 2004; Zhao et al. 2004; Impa et al. 2005; Centritto et al. 2009). Genotypic variation has been noted for $\delta^{13}C$ or

CID values in rice. The japonica genotypes show higher $\delta^{13}C$ values or lower CID values than the indica ones (Takai et al. 2009; Xu et al. 2009; This et al. 2010). Kano-Nakata et al. (2014) proclaimed that the $\delta^{13}C$ value of panicles may be the best indicator of plant water status among various plant organs in rice under drought.

6.3 Targeting Cuticle in Minimizing Water Loss

Plants utilized different mechanisms like increasing wax deposition in the cuticle, reducing the number of stomata per unit area, presence of trichomes, reduction in leaf size, and disposition of leaves with respect to the incident radiation to reduce water loss. Islam et al. (2009) reported that improved drought tolerance has also been related to increased levels of cuticular waxes in rice. González and Ayerbe (2009) confirmed the link between drought tolerance and cuticle properties by breeding for improved water use efficiency, tolerance, and yield under water deficit conditions which caused increased amounts of cuticle waxes. The presence of a prominent epicuticular wax layer increases WUE by reducing cuticular transpiration and increasing leaf boundary layer effects, and it decreases leaf and canopy temperatures by reducing the net radiation reflecting solar radiation (Jefferson et al. 1989).

Drought stress causes to mentose or waxy leaves of some plants are both of these characteristics are reflected by the increasing amount of leaves to reduce water loss (Leila 2007). Thus, the increased biosynthesis of cuticle waxes seems to be an established plant response to dry conditions. Zhou et al. (2013) carried out a functional analysis of OsGL1-6 in rice, homologous to CER1 in *Arabidopsis* and Wda1 in rice, widely expressed in vegetative and reproductive organs, and especially highly expressed in leaf epidermal cells and vascular bundles. A phenotypic characterization and drought sensitivity experiments on OsGL1-6 antisense-RNA transgenic plants showed that OsGL1-6 is involved in cuticular wax accumulation and drought resistance. Thus, genetic modification of OsGL1-6 may have great possibilities for advancement of the drought resistance in rice.

6.4 Improving Production Efficiency

The WUE of plants can be enhanced by genetic modification of plants for higher harvest index, spikelet fertility, stay-green traits, seedling vigor, and short duration and reduced photorespiration. Now, QTLs have been identified for some of these traits in rice, and efforts are being made to identify the genes and understand the molecular basis. Molecular markers that are linked to QTLs that control panicle sterility and reproductive traits (Lanceras et al. 2004) have been identified in rice. The WUE is a complex trait and determination of WUE under field condition needs an accurate measurement of water and carbon budget of plants. During photosynthesis, plants with high water use efficiency show less discrimination to $^{13}CO_2$ and $^{12}CO_2$. Hence, ^{13}C-isotope discrimination by photosynthesis has been successfully established as a surrogate for WUE. Biotechnological options for enhancing water

use efficiency of rice have been observed to be associated with low ^{13}C-isotope discrimination in rice (Peng et al. 1998). Identification and molecular marker tagging of QTLs controlling $^{13}CO_2$ discrimination and application of this approach in rice will, therefore, help in enhancing WUE. A key factor in determining plant productivity under drought conditions is water use efficiency (WUE), and it is mentioned as a strategy to improve crop performance under water-limited conditions (Araus et al. 2002). Agronomic parameters like photosynthetic rate, relative water content (RWC), and stomatal conductance show strong positive correlations with WUE, whereas transpiration rate expresses negative correlation with WUE under drought in rice varieties (Akram et al. 2013).

6.5 Improving Tolerance Mechanism to Limited Water Condition

Cellular water deficit stress tolerance in plants depends upon the expression of genes involved in the production of organic compatible solutes (proline, sugars, polyols, betaine, etc.), late embryogenesis abundant (LEA) proteins, and antioxidants. They protect plants from stress by (1) osmotic adjustment which helps in turgor maintenance, (2) detoxification of radical oxygen species, (3) stabilization of the quaternary structure of proteins, and (4) allowing the plant to extract water at low soil water potential. Osmotic adaptation is the accumulation of solutes in cells due to maintaining cell volume against water loss under stress conditions (Heidaiy and Moaveni 2009). During the water stress, cellular ROS production increases; simultaneously, a number of enzymatic and nonenzymatic antioxidants in chloroplasts protect the cell by controlling the intracellular ROS concentration (Srivalli et al. 2003). The peroxidation of lipids in the cell membrane is one of the most damaging cellular responses observed in response to water stress (Thankamani et al. 2003). Vikram et al. (2011) noted that a major QTL, *qDTY1.1*, containing three rice populations, N22/Swarna, N22/IR64, and N22/MTU1010, performed significant grain yield at the reproductive stage drought.

Transcriptome engineering or overexpression of a master switch gene (such as stress sensors, protein kinases, or transcription factors) that regulates several target genes coding for osmolyte biosynthesis enzymes and LEA proteins is a potential tool to adapt abiotic stresses. Transgenic rice plants are enabled to show abiotic stress tolerance including drought by overproducing organic solutes such as glycine betaine (Sakamoto and Murata 1998), proline (Su and Wu 2004), and trehalose (Garg et al. 2002). Transgenic overexpression of a stress-inducible calcium-dependent protein kinase (OsCDPK7) in rice increased cold, salinity, and drought tolerance (Saijo et al. 2000). In addition to that, QTLs that confer osmotic adjustment have been reported in rice using molecular markers (Zhang et al. 2001). Xu et al. (2008) carried out an experiment of overexpression of zinc finger protein OsZFP252 in rice. They reported increases in soluble sugar and proline accumulation and also 74–79% higher chances of survival by enhancing drought tolerance. Many reports showed that under drought stress, a cellular water deficit is occurring that leads to the accumulation of LEA proteins. Xiao et al. (2007) showed overexpressing the

encoding LEA gene OsLEA3-1 in rice enhanced drought tolerance in the field response to water deficit stress. Similarly, Duan and Cai (2012) reported that overexpression of OsLEA3-2 proteins in rice also showed drought tolerance and the yield loss was less compared to control treatment under a severe drought field condition. When the HVA1 (gene-encoding LEA protein) from barley was overexpressed in rice, there was a significant increase in growth performance and water use efficiency under drought stress (Babu et al. 2004). The *Arabidopsis* DREB1A transcription factor controls the expression of several *LEA* genes and genes code for osmolyte biosynthesis enzyme. Many authors reported that overexpressing of *DREB1A* gene in transgenic rice plants has been shown to improve drought stress tolerance (Oh et al. 2005; Ito et al. 2006; Datta et al. 2012). Under drought conditions, *SNAC1* gene is produced substantially in guard cells and encodes a NAM, ATAF, and CUC (NAC) transcription factors with transactivation activity. Overexpressed *SNAC* genes such as *SNAC1*, *OsNAC6*/*SNAC2*, and *OsNAC5* exhibited significant improvement of drought tolerance in rice (Hu et al. 2006; Takasaki et al. 2010; Nakashima et al. 2014).

Transgenic rice lines have been developed using many transcription factors with either constitutive or inducible promoters, such as HvCBF4 (Oh et al. 2007), AP37 (Kim and Kim 2009; Oh et al. 2009), TaSTRG (Zhou et al. 2009), OsNAC045 (Zheng et al. 2009), ERF protein TSRF1 (Quan et al. 2010), ERF protein JERF3 (Zhang et al. 2010), OsDREB2A with the 4ABRC promoter (Cui et al. 2011), OsDREB2A with the rd29A promoter (Mallikarjuna et al. 2011), SbDREB2 (Bihani et al. 2011), OsSD1R1 (Gao et al. 2011), OsDREB1A and OsDREB1B (Datta et al. 2012), AtDREB1A (Hussain et al. 2014; Ravikumar et al. 2014), *OsNAC6* (Rachmat et al. 2014), and the bZIP family (Xiang et al. 2008; Liu et al. 2014). Overexpression of transcription factor HYR (higher-yield rice) increases photosynthesis leading to higher grain yield in rice under drought conditions (Ambavaram et al. 2014). Thus, transcription factors are the main regulators of gene expression and could be selected as primary targets for biotechnological engineering to develop stress tolerance in rice plants.

7 Breeding Strategies for NUE and WUE

7.1 Selection of Germplasm

Genotype selection indirectly in the selection environment could yield better results. But the selection environment needed to be similar to that of the target environment where genotype will be cultivated. The N stress level should be imposed accordingly. In moderately N stressed target environment, indirect selection might be allowed where there is a similarity between selection and target environments (Cormier et al. 2013), whereas selection under water deficit environment or at rainfed condition will allow for selecting water-saving rice cultivar. Genotypes that showed better NUE might have some mechanism to combat the limited N level. Apart from this, the crop duration can be considered in selecting the genotype

as long-duration genotypes will require more N for growth and development and for ultimately producing more yields for an extended period of time. In addition, environment x N interaction study would be best breeding strategy for selecting cultivar having more NUE in crop plants including rice. Similarly, genotype x environment interaction study under different water-limited environments would identify better cultivar with elevated WUE in rice. Alternatively, germplasm selection based on secondary traits associated with drought tolerance or escaping mechanism might improve the performance under water-limited environments (Liu et al. 2010).

7.2 Heterosis Breeding

Exploiting heterosis to confer NUE in crop plants could be a good option for plant breeders. Hybrids could perform in expressing particular characters for NUE (Cormier et al. 2016). Studies reported that best parent heterosis was consistent in field plots for grain yield in different hybrids (Borghi et al. 1988; Brears et al. 1988; Morgan et al. 1989). Higher N uptake and NUE in hybrids were assumed than pure lines (Cormier et al. 2016) from a study by Perezin et al. (1998) and Oury et al. (1994, 1995). Kindred and Gooding (2005) reported heterosis in hybrids for total N in aboveground biomass at high N level, whereas best parent heterosis at low N level for total N at grain filling phase was reported by Le Gouis and Pluchard (1996) and Le Gouis et al. (2002). Heterosis for different root traits was concluded in different studies (Kraljevic-Balalic et al. 1988; Wang et al. 2006). However, heterosis can be exploited for better NUE in rice.

In water-limiting condition, attaining optimum production of rice becomes challenging due to drought effect (Dwivedi and Pandey 2012). Shortage of water is an important hindrance in rice-growing areas in Asia, and that forced breeders to work on developing less water requirement cultivars (Zhao et al. 2008). There is scope to develop such cultivar through exploiting appropriate breeding strategies. Sources of genes conferring improved performance under water-limited environment might be landraces and wild relatives (Xia et al. 2006). Transferring such characters to the cultivated variety or developing new variety incorporating particular genes is possible in modern breeding. Exploiting heterosis to develop new cultivar with such genes through hybridization program (Srivastava 2000) will offer better water use efficiency. In a study, improved resistance to drought was reported for hybrids (Ushakumari et al. 2014). Hybrids also performed better than the parents in terms of yield and related traits under drought environment (John Kingsly et al. 2018).

8 Molecular Approaches for NUE and WUE

8.1 Association Mapping Based on Panels

Genome-wide association studies (GWAS) had been employed by the researchers to dissect complex quantitative traits. GWAS generally provides high resolution based on recombination events that involve a large panel of genotypes from diverse sources. Studies on root-related traits are much lesser than the aboveground traits. Moreover, relatively low heritability of these traits reduces the statistical power studying the association genetics. This problem could be resolved by increasing the panel size, but it is associated with large experiments with more NUE traits which make the study expensive (Hawkesford and Griffiths 2019). Despite the particular problem, quite a few studies were carried out by researchers in many crops such as maize (Zaidi et al. 2016) and wheat (Cormier et al. 2014). Though there are lots of effort that have been made for detecting genomic regions related to different traits, only few attempts were for WUE associated traits in rice (Vasant 2012; Courtois et al. 2013; Muthukumar et al. 2015). A number of 80 marker trait associations were identified for yield, height, and flowering traits in rice, of which some were associated with drought stress-responsive (Swamy et al. 2017; Norton et al. 2018; Alshugeairy 2015).

8.2 QTL Mapping Using Biparental Populations

One of the long established methods of detecting traits associated with genomic regions is QTL mapping. It involves the segregating population, as well as fixed homozygous lines (RILs and DH), derived from two parents. The produced population bears the same genetic information from only two sources which increases the statistical power of these tools (Hawkesford and Griffiths 2019). A number of NUE traits related to QTLs were identified in rice (Wei et al. 2011). QTL mapping approach has successfully been used for detecting QTLs related to WUE-related traits. Quite a large number of QTLs for WUE traits have also been identified in rice (Ashraf 2010; Bernier et al. 2008; Swamy et al. 2014, 2017; Thomson et al. 2010); few of them were fine-mapped (Bernier et al. 2007, 2009a, 2009b; Salunkhe et al. 2011; Boopathi et al. 2013; Mishra et al. 2013; Solis et al. 2018).

8.3 Multiparent Populations (MPP)

Researchers around the world try to make best use of GWAS and QTL mapping using a multiparent population (*MPP*). Nested association mapping (*NAM*) in maize (Yu et al. 2008) and multiparent advanced generation intercross (*MAGIC*) in wheat (Mackay et al. 2014) and in rice (Bandillo et al. 2013) are the success story of this approach. The restrictions of the use of a single approach can be overcome by using next-generation technologies in the era of the changing climate. Moreover, recent

technological advances in post-omics era markedly reduced the cost of genotyping which allows researchers to explore latest tools and strategies to analyze more data points to dissect underlying genetics behind the complex traits. Particularly after the availability of reference genome of rice, research based on genomics boosted up in identifying new genetic variations due to exploitation of high-throughput genotyping with the association of precision phenotyping. Attempts might be made involving MPP to dissect the underlying genetics regarding NUE and WUE in rice.

8.4 Backcross Populations (BC)

Backcross populations are of a point of interest as it carries most of the genetic information of recurrent parent. The rest of the genetic materials are from the donor parent. The desired trait(s) is donated by donor parents. The process of backcross involves a crossing between recurrent and donor parents having targeted traits and then subsequent backcrossing of the progeny with the recurrent parent. This will ensure the composition of recurrent parent's genetic constituents to the segregating populations more than the donor parents. NUE and WUE associated traits can be transferred by this technique more accurately to the recurrent parents. This can then be compared with phenotyping of both parents and the progeny for confirmation. Keeping the whole genotyping information in each generation could be accumulated to build a library which can then be utilized in further genomic studies as a reservoir of chromosomal segment information at different backcrossing generations. This approach could be a good option in rice cultivar development containing QTL for NUE and WUE traits. This particular strategy had been adopted to study phosphorus efficiency in barley by Soleimani et al. (2017). Marker-assisted backcrossing could play important role in developing quality rice cultivar by incorporating the gene of interest into elite variety (Hasan et al. 2015). Surapaneni et al. (2017) identified 15 QTLs for agronomic traits and a set of 74 chromosomal segment substitution lines (*CSSLs*) from backcross inbred lines. Jewel et al. (2019) developed a total of 230 BC1 F5 lines from a backcross which was utilized in identifying QTL for NUE in rice, and 22 QTLs were identified in different genomic regions which were involved in nutrient uptake from soil. Sun et al. (2017) employed four advanced backcrosses overlapping populations to dissect panicle components in rice. Xia et al. (2017) used backcross population derived from slender *indica Jin23B* and round *japonica QingGuAi* for grain shape study. A number of 325 BC2F2 bulk populations were developed from huge backcrossing to improve drought stress tolerance in rice (Lafitte et al. 2006). Thirteen QTLs for drought tolerance were detected from 72 introgression lines developed from backcross populations (Cui et al. 2018). BC1F2-derived introgression lines showed better yield potentiality under water-limited environment (Anyaoha et al. 2019).

8.5 Near-Isogenic Lines (NILs)

Near-isogenic lines (NILs) are the progeny from a cross between a recurrent parent and a donor parent whose genetic constituents are the same as recurrent parent apart from few genes or locus which are from donors (Young et al. 1988). NILs are generally developed for validating a QTL for a trait of interest. NIL populations had a homogeneous genetic background except for a small genetic fragment. The phenotypic dissimilarities within the population are probably due to the regions of marker locus. Near-isogenic populations have been utilized in breeding programs for developing new cultivars and for genomic analysis in many crops (Edwards et al. 2005; Blanco et al. 2006). The entire genetic information of each generation holds significance in resistance breeding against biotic and abiotic stresses as well as in many agronomic traits. A number of study have been successfully carried out in rice involving NILs to dissect complex traits including lowland drought tolerance (Venuprasad et al. 2011), stresses (submergence, lodging, salinity) at coastal areas (Reddy and Rani 2018), rice blast resistance (Jain et al. 2017), *BPH* resistance (Jena et al. 2017), rice yellow mottle virus (*RYMV*) resistance (Taylor and Jalloh 2017), and root morphology and grain yield (Selvi et al. 2015). This approach could also be proposed for NUE and WUE associated traits in rice.

9 Conclusion

From the above discussion of this chapter, it is revealed that phenotypic traits such as root, especially root length, density, and depth, have long been seen as critical traits in order to improve N and water use efficiency and crop adaptation in nonoptimal environmental conditions. The size and activity of the root system determine the rate at which the shoot system can produce photosynthates. It is evident that improving yield under stress conditions will require a whole-plant approach. With the ongoing research and progress, breeding of new varieties with improved ability and capability to use N efficiently and/or to produce more despite water scarcity becomes possible. Those varieties will reduce the application of N fertilizer to reduce environmental pollution and cost of production. Similarly, tolerance varieties will facilitate water management programs and perform better WUE and water productivity that enable rice adapt to environments under climate change.

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Aromatic Rice: Biochemical and Molecular Basis of Aroma Production and Stress Response

Puja Ghosh and Aryadeep Roychoudhury

Abstract

Aromatic rice constitutes one of the most demanding staple food crops all over the international market owing to its strong flavor which is a result of a number of volatile compounds, among which 2-acetyl-1-pyrroline (2-AP) is the principal one. The 2-AP biosynthetic pathway and related metabolites and enzymes are therefore very important for understanding the probable cause of aroma production. The key gene responsible for the aroma production in rice is *betaine aldehyde dehydrogenase 2* which results in the inhibition of gamma-aminobutyric acid (GABA) production and leads to the accumulation of Δ^1-pyrroline, the immediate precursor of 2-AP in aromatic rice, by causing a 8 bp deletion in its seventh exon. A number of mutations have been reported in different exons and introns of *BADH2* gene, which needs to be studied. Many molecular marker systems like the allele-specific amplification (ASA) and cleaved amplified polymorphic sequences (CAPS) have been developed concerning the mutations in the exons of *BADH2* gene. Many molecular markers have also been developed to identify the aroma locus. Overexpression and suppression of genes like *P5CS* and *BADH2* are known to increase aroma production in rice. Environmental factors and climate have also been reported to play an important role in the aroma production of rice cultivars. Exogenous applications of few metabolites have the potentiality to increase the aroma content in rice cultivars. Impact of growth regulators on aromatic cultivars has also been studied. Aroma has also been reported to vary in various stages of development in rice like the vegetative, reproductive, grain-filling, and mature grain stages. The present chapter provides a broad overview of the biochemical and molecular basis of aroma production in rice.

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Keywords

Aromatic rice · Aroma volatiles · Stress response · Betaine aldehyde dehydrogenase 2 · Biochemical and molecular markers · Seed developmental stages

1 Introduction

The great demand for flavor and fragrance in the world is due to volatile compounds released from plants. Fragrant flavor of aromatic rice is a special trait which helps in their popularity in the world market owing to their huge demand, thus making them economically significant (Nadaf et al. 2014). Scented rice varieties are well-known for specific "nutty popcorn"-like flavor which is released during the time of cooking. Presence of 200 or more volatile compounds including hexanal, octanal, nonanal, 4-vinylphenol, and 4-vinylguaiacol are known to impart characteristic flavor to aromatic rice varieties. Among the mentioned compounds, 2-acetyl-1-pyrroline (2-AP) is considered as the prime component that imparts aroma to the fragrant rice cultivars (Ghosh and Roychoudhury 2018). 2-AP is found in all plant parts of the scented varieties except the roots and mainly concentrated in higher amounts in the aerial segments when compared to the milled grains (Chen et al. 2008). Ahn et al. (1992) was successful in localizing a gene (*fgr*) on the long arm of chromosome 8 which was found to be responsible for the aroma formation. This gene was tagged as major and recessive quantitative trait loci (QTL) in the same region but was limited to a genetic distance of 12 cM in a (IR-64 × Azucena) doubled haploid (DH) population, where the variety Azucena was used as a donor for aroma trait (Lorieux et al. 1996). In recent times, Bradbury et al. (2005) further specified the aroma region which led to the identification of a single recessive gene responsible for fragrance. Aroma in fragrant cultivars mainly occurs due to an 8 bp deletion and three single-nucleotide polymorphisms (SNPs) in the exon 7 of the *betaine aldehyde dehydrogenase 2* (*BADH2*) gene of scented rice cultivars. The mutation leads to a frame shift that creates a premature stop codon that leads to the non-functionality of the *BADH2* gene (Bradbury et al. 2005). Several other mutations in other exons and introns of *BADH2* gene have also been reported previously (Kovach et al. 2009; Sakthivel et al. 2009). Development of different molecular markers, for example, allele-specific amplification (ASA) and cleaved amplified polymorphic sequence (CAPS) markers, has been made so as to track down these mutations (Bradbury et al. 2005; Dissanayaka et al. 2014). The mutation in the *BADH2* gene leads to the non-functionality of the enzyme in the aromatic cultivars that inhibits the natural formation of gamma-aminobutyric acid (GABA) from gamma-aminobutyraldehyde (GABald) and leads to the cyclization of Δ^1-pyrroline in the fragrant cultivars which is the immediate precursor of 2-AP (Ghosh and Roychoudhury 2018).

Apart from 2-AP, different other volatiles are present whose contribution to the total aroma level is undeniable (Sukhonthara et al. 2009). Study of volatile profiles during developmental growth of aromatic rice cultivars reveals the significant changes in the volatile organic compounds (VOCs) (Hinge et al. 2016).

Plant growth regulators are also known to have significant impacts on the aromatic cultivars (Goufo et al. 2011). Improvement in the quality of aroma in fragrant cultivars can be induced by aroma-promoting factors like salinity stress, drought stress, and reduction of solar energy (Sansenya et al. 2017). Elements like zinc, lanthanum, and manganese are also reported to enhance aroma (Mo et al. 2016; Li et al. 2016). Gene manipulation techniques and development of different transgenics have also led to improved quality of aroma in aromatic rice as well as development of aromatic cultivars from the non-aromatic ones (Kaikavoosi et al. 2015; Niu et al. 2008).

Owing to its popularity in the world market, aromatic volatiles that impart the characteristic flavor to the fragrant cultivars are being studied extensively, and experiments to understand the nature of aroma trait so as to develop varieties with the desired aroma quality are being continuously performed. The present chapter aims at providing an overview of the information available till date with regard to the aroma trait in aromatic rice.

2 Mutations Reported in the *BADH2* Gene

Discovery of the *BADH2* gene responsible for aroma production and presence of a large fragrant rice gene pool in rice has led to the search for allelic variants at the *BADH2* locus (Sakthivel et al. 2009). *BADH2* gene comprises of 15 exons and 14 introns that code for 503 amino acids. Some of the major mutations reported are mentioned in the table below:

Type of mutation	Reference
G → T substitution in the splice donor site at exon 1-intron 1 junction	Ootsuka et al. (2014)
C → T substitution in exon 13	Ootsuka et al. (2014)
Absence of miniature interspersed transposable element (MITE) in promoter	Bourgis et al. (2008)
7 bp deletion in second exon	Shi et al. (2008); Kovach et al. (2009)
3 SNP and 8 bp deletion in seventh exon	Bradbury et al. (2005)
2 bp deletion in first exon	Kovach et al. (2009)
1 bp insertion in tenth exon	Kovach et al. (2009)
G → T substitution in tenth exon	Kovach et al. (2009)
1 bp deletion in tenth exon	Kovach et al. (2009)
1 bp insertion in 14th exon	Kovach et al. (2009)
3 bp insertion in 13th exon	Myint et al. (2012)
G → T substitution in 14th exon	Kovach et al. (2009)
C → T substitution in 14th exon	Kovach et al. (2009)
7 bp insertion in exon 8	Amrawathi et al. (2008)
2 SNPs in central region of intron 8	Sun et al. (2008)
TT deletion in intron 2	Chen et al. (2008)

(continued)

Repeated $(AT)_n$ insertion in intron 4	Chen et al. (2008)
803 bp deletion between exons 4 and 5	Shao et al. (2011)
C → A SNP in exon 10	He et al. (2015)
3 bp deletion in exon 12	He et al. (2015)
C → G SNP in exon 1	He et al. (2015)
C → T SNP in exon 5	He et al. (2015)
8 bp insertion in the promoter	Bindusree et al. (2017)

3 Biochemical Markers for the Discrimination of Aromatic Cultivars

India has passed a sui generis legislation named Protection of Plant Varieties and Farmers' Right Act 2001 (PPVFR) in order to protect plant varieties by registering them. Since registration of plant varieties have gained much attention and significance all over the world (including India), DUS (distinctiveness, uniformity, and stability) testing becomes mandatory for carrying out the procedure of varietal registration. Though DUS guidelines asks for morphological descriptors, due to the increasing number of varieties among the plants, it might become difficult to identify the varieties solely based on morpho-physiological characteristics; hence the mentioned study used biochemical and molecular markers for the identification of varieties (Patra and Chawla 2010). Patra and Chawla (2010) conducted a study of 18 traditional and crossbred Basmati rice varieties for the DUS testing. Electrophoretic profiles of isozymes and proteins are considered as potential biochemical markers for identifying crop varieties. Their investigations revealed the presence of 24 polypeptide bands, out of which 10 were polymorphic (moderate). Unweighted pair group method with arithmetic mean (UPGMA) cluster analysis could efficiently differentiate nine varieties, viz., Bas-98/69-7-2, Super Basmati, Kastoori, PSD-17, Taraori Basmati, Tapovan Basmati, Type 3, Basmati-370, and Pusa Basmati-1. Hansraj and Hansraj-3078 exhibited 100% resemblance and so did Yamini and Basmati-386 which shared a common parent, Pakistani Basmati-1 and two Basmati restorer lines, Pusa Sugandh-2 and Pusa Sugandh-3. In the mentioned study, five enzymes, viz., superoxide dismutase (SOD), aldehyde dehydrogenase (ADH), malate dehydrogenase (MDH), esterase (EST), and peroxidase (POX), were selected for conducting the isoenzyme profiling of the varieties under investigation. Among the five mentioned enzymes, SOD exhibited monomorphic banding patterns for all the Basmati varieties under study. The other four enzymes exhibited moderate to higher degree of polymorphism. Super Basmati was the single cultivar among the other varieties which exhibited two bands of ADH-1 and ADH-2. Hansraj-3078 was separated from Hansraj by the absence of the band of MDH-4 in the latter. Pusa Sugandh-2 exhibited the presence of MDH-2 and MDH-4 bands, while Pusa Sugandh-3 lacked both the bands. Kastoori and Lal Basmati exhibited the presence of a dark band of EST-3, while it was found to be absent in the other cultivars. Pusa

Sugandh-3 showed a dark band of POX-3, while Pusa Sugandh-2 showed a lighter band of POX-3. UPGMA cluster could easily distinguish all the varieties under investigation except Hansraj as it exhibited 100% similarity to KLS-24, whereas it registered only 93.3% similarity with its derived variety, Hansraj-3078.

4 Molecular Markers

The inheritance of aroma was reportedly controlled by 1–3 dominant or recessive genes specifying the complicated dominance of the trait at the genetic level. Such disputing knowledge on inheritance of aroma was mainly due to segregation distortion, undependable and inefficient phenotyping methods used for deduction of aroma quality, and failure to understand the aroma of endosperm in rice. Experiments to map the aroma trait at molecular level have been attempted. Efficient techniques like high-density mapping of molecular markers and sequences in rice genome have further permitted fine mapping and positional cloning of the desired gene (Sakthivel et al. 2009).

4.1 Simple Sequence Repeat (SSR) Markers

Simple sequence repeat (SSR) is significant equipment in modern world for detection of genetic variance among cultivars. SSR is a very efficient tool with merits like quickness, simplicity, rich polymorphism, and stability. The mentioned features are widely used for analysis of genetic diversity, gene mapping, fingerprint construction, conduction of gene purity test, and utilization in heterosis specifically in detecting species with closer genetic relationship (Sajib et al. 2012).

Sajib et al. (2012) studied the molecular characterization of 12 elite aromatic rice varieties using 24 SSR markers, among which nine microsatellite markers showed polymorphism. The range of number of alleles per locus was 2 (RM510, RM244, RM277) to 6 (RM163) with an average of 3.33 alleles across nine loci obtained in the study. RM163 was considered as the best marker in the study for identifying the 12 genotypes, depending on their respective PIC (polymorphism information content) values. It was noted that the frequency of the most common allele ranged between 41% (RM163, RM590, and RM413) to 91% (RM510). The highest genetic distance was calculated among the following pair of rice cultivars, viz., Basmati PNR 346 and Deepa, Basmati PNR 346 and Patnai-23, Dolargura and Sugandha, Bhogganijia and Sugandha, and finally Dolargura and Chinikani. Though Opchaya, Basmati PNR 346, and Sugandha showed close similarity among themselves, it was noted that they were quite dissimilar when compared with the other varieties. The mentioned study thus revealed that Dolargura and Opchaya could be used as potential parents for breeding programs conducted for the improvement of fine grain aromatic rice cultivars owing to the fact that both of them have been placed in distant clusters in the given study.

4.2 Amplified Fragment Length Polymorphism (AFLP) Markers

Amplified fragment length polymorphism (AFLP) is a reproducible technique for studying genetic variation and has gained popularity due to its features like no need for prior sequence information of the genome under investigation. It is popular for providing the best genome-wide coverage and also bears the quality amenable to semi-automated genotyping. AFLP holds an important position in plant research due to its utility in the study of genetic diversity, varietal identification, mapping, quantitative trait loci analysis, and gene isolation (Agarwal et al. 2002).

Agarwal et al. (2002) used the fluorescence-based AFLP (f-AFLP) technique to compare 33 rice genotypes including traditional Basmati, Basmati-like, and non-aromatic cultivars to study their interrelationship and genetic diversification so that the feasibility of obtaining individual genotypic specific profiles can be documented. The scored data points indicated toward the total number of AFLP markers to be 501, among which 65% of the markers exhibited polymorphism. The fluorescent AFLP markers helped in determining the presence of considerable amount of genetic variability which exists in genotypes under study. It also helped in concluding the fact that traditional Basmati rice varieties could easily be distinguished from the crossbreed Basmati-like genotypes as well as the non-aromatic cultivars. The study also indicated that crossbreed Basmati varieties from the subcontinent or somewhere else were found to be genetically quite distant. In general, f-AFLP-based clustering helps in concluding the putative degree of improved genotypes.

4.3 Restriction Fragment Length Polymorphism (RFLP) Markers

Restriction fragment length polymorphism (RFLP) is a molecular technique which can be used to detect foreign gene introgression and helps in carrying out marker-based selection of genes of interest and preparation of clones with unknown products (Jena et al. 1992). Ahn et al. (1992) identified a DNA marker which was closely linked to the gene of aroma by examining 126 mapped rice genomic and cDNA and oat cDNA clones as hybridization probes used in Southern blots containing DNA from a pair of nearly isogenic lines either with the aroma gene or devoid of the aroma gene. Hybridization of the chromosomal segments from the genome of the donor was easily distinguished by RFLPs between the nearly isogenic lines (nils). The presence of linkage among the gene and the DNA marker was indicated by the co-segregation of fragment phenotype and the allele derived by the donor. Thus, the study exhibited the close linkage of the *fgr* gene with the single copy clone RG28 on chromosome 8 and indicated the future usage of this marker for indicating the presence or absence of fragrance along with an efficient detection of the genotype being homozygous or heterozygous.

4.4 Randomly Amplified Polymorphic DNA (RAPD)

Randomly amplified polymorphic DNA (RAPD) is a very efficient tool which accelerates the process of gene mapping. The technique involves the PCR-based amplification of DNA with random decamer. RAPD analysis has proven its efficiency in construction of genetic maps for plants (Nematzadeh et al. 2004). Michelmore et al. (1991) demonstrated the efficiency of RAPD when combined with bulk segregant analysis and indicated toward detection of identifying markers linked to a specific gene or a specific region of genome.

Nematzadeh et al. (2004) reported the mapping of an aromatic gene with the help of RAPD analysis via bulk segregation analysis (BSA) in the F_2/F_3 population of Basmati 370 and IR-36. DNA samples from homozygous aromatic and homozygous non-aromatic variety were identified with the help of progeny test and were bulked for BSA. Among a total of 550 primers, AG8 and AN1 exhibited polymorphism and helped in distinguishing between the aromatic and non-aromatic varieties. The gene of aroma was found to be associated with three primer pairs, viz., AG8-AR, AN1-AR1, and AN1-AR2 by surveying the F_2 generation. The mentioned primer pairs were found to be linked with the gene for aroma and hence mapped with a distance of 6.9 (AG8-AR), 8.9 (AN1-AR1), and 16.4 (AN1-AR2). Linkage between the gene for aroma and primer pair AG8-AR was confirmed by Southern blotting using the AG8-AR as the probe, and hence AG8-AR was mapped on chromosome 8 flanked tightly by linked markers RZ617 and RG978 at 2.1 and 1.7 cM distance, thus indicating the position of the gene on chromosome 8.

4.5 Cleaved Amplified Polymorphic Sequences (CAPS)

Cleaved amplified polymorphic sequences (CAPS) marker is an important tool in the field of molecular biology for the genetic and breeding studies of plant species. The principle of CAPS marker includes a polymerase chain reaction with primers designed for a specific region followed by restriction digestion of amplicons by endonuclease and finally separation of the fragments on agarose gel. The endonuclease is the key component in the determination of polymorphism in the plants (Shavrukov 2016).

Bradbury et al. (2005) reported that most of the aromatic rice cultivars, namely, Basmati and Jasmine, contain a mutated exon 7 of *BADH2* gene with an 8 bp deletion and three SNPs. Kovach et al. (2009) named the allele as *badh2.1*. The mentioned allele creates a premature stop codon which further produces a non-functional BADH2 enzyme that leads to the accumulation of 2-AP, the key component contributing fragrance in aromatic rice (Dissanayaka et al. 2014). Some Sri Lankan rice varieties exhibited the absence of *badh2.1* allele. Thus, Dissanayaka et al. (2014) found a "G insertion" in the 14th exon of *BADH2* gene in some exotic Sri Lankan rice cultivars, which was named *as badh2.7* allele in the fragrant varieties originated in Sri Lanka. A noble CAPS marker was developed to detect the *badh2.7* allele with the amplicon of exon 14 of *BADH2* gene and followed by restriction

digestion of the same by *Bsl*I enzyme. The CAPS marker was designed to detect the "G" insertion in the 14th exon amplicon product, and the *Bsl*I enzyme cleaves at 14th, 269th, and 336th bp, thus producing four fragments of size 14, 255, 67, and 121 bp in the wild-type varieties. The scented rice cultivars bearing the "G" insertion have the 336th bp restriction site deleted and hence result in three fragments of size 14, 255, and 188 bp. Among the 20 varieties of exotic-aromatic, aromatic, and non-aromatic varieties, only four Sri Lankan cultivars exhibited the 188 bp band, viz., Suwanda A1, Kuruluwee, and Suwadal. In all other rice varieties including Basmati, like Pusa Basmati 1, Basmati-370, Basmati 217, and Basmati Lamo, along with three Sri Lankan varieties, the aromatic product fragrant alleles at seventh exon were confirmed by the allele-specific amplification marker, developed by Bradbury et al. (2005) except Kuruluthuda and Heenati, where none of the mutations were noticed. This further leads to the conclusion that the fragrance of these two cultivars might be due to some other exon mutation in *BADH2* gene. The assumed protein structure for this mutant exhibited partial loss of oligomerization and coenzyme-binding domain. Thus, the BADH2.7 CAPS marker developed in this study can help in future breeding of aromatic rice as well as might be useful in marker-assisted selection.

4.6 Allele-Specific Amplification

Allele-specific amplification (ASA) is a cost-effective and robust tool that can be used to distinguish alleles that differ by SNPs and mutations like deletions or insertions. Bradbury et al. (2005) reported that ASA assay is a single-tube PCR-based analysis which helped in discriminating the fragrant and non-fragrant cultivars. It also helped in detecting homozygous fragrant, heterozygous non-fragrant, and homozygous non-fragrant genotypes among a population segregation done for the fragrance trait. The investigation involved the designing of two types of external processes, viz., external sense primer (ESP) and external antisense primer (EAP), which acts as internal positive control and amplifies a region of approximately 580 bp in both scented (577 bp) and non-scented (580 bp) genotypes. The designed internal primer IFAP (internal fragrant antisense primer) and INSP (internal non-fragrant sense primer) bind with their corresponding external primers, ESP and EAP. The use of four primer combinations can lead to three probable outcomes, viz., a 580 bp positive control band; when a band of 355 bp is produced along with the positive control band, it clearly indicates toward the cultivar being homozygous non-fragrant. When a 257 bp band appears along with the positive control band, the result indicates that the variety is homozygous fragrant. When both the 355 bp and 257 bp appear with one positive control, the genotype is concluded to be heterozygous non-fragrant. In this assay 168 F_2 progeny were segregated for fragrance with 100% efficiency which includes 46 homozygous fragrant, 80 heterozygous non-fragrant, and 42 homozygous non-fragrant. Thus, ASA can be used to screen rice across a wide range of rice varieties and within segregating populations

and is easy method to determine homozygosity or heterozygosity of a rice related to aroma trait.

5 Biochemistry of Aroma

The metabolic pathway of aroma production is still under vivid study. The work done so far to decipher the pathway states the presence of three amino acids as significant precursors in the biosynthetic pathway of aroma, viz., proline, ornithine, and glutamate. The precursor amino acids play an important role in the formation of an intermediate Δ^1-pyrroline-5-carboxylate with the help of their respective catalyzing enzymes, viz., proline dehydrogenase (PDH), ornithine aminotransferase (OAT), and Δ^1-pyrroline-5-carboxylate synthetase (P5CS) (Huang et al. 2008). Putrescine was also suggested to play a vital role toward aroma production (Vanavichit and Yoshihashi 2010) since Δ^1-pyrroline, which is a product formed as a result of proline catabolism, through putrescine oxidation is the immediate precursor of the pyrrole ring of 2-AP (Costello et al. 2001). Diamine oxidase (DAO) is known to catalyze the conversion of putrescine to gamma-aminobutyraldehyde (GABald), which further leads to the formation of gamma-aminobutyric acid (GABA) or cyclises to form Δ^1-pyrroline (Gill and Tuteja 2010) depending on the presence or absence of functional BADH2 enzyme (Nadaf et al. 2014). Methylglyoxal is the key metabolite which is known to react non-enzymatically with the metabolites leading to the production of aroma (Huang et al. 2012).

Ghosh and Roychoudhury (2018) investigated the role of the metabolites and enzyme activities leading to the formation of aroma in fragrant cultivars of rice using 11 aromatic and 4 non-aromatic cultivars. The study highlighted the increment in the level of metabolites like proline, methylglyoxal, and intermediate compound P5C and enhanced activities of enzymes like P5CS, PDH, OAT, and DAO in the aromatic cultivars compared to the non-aromatic ones, thereby proving their role in the formation of aroma in the fragrant cultivars. Simultaneous reduction in the GABA content along with the lowering of the BADH2 activity in the aromatic cultivars further confirms their vital functions in the pathway of aroma formation. A reverse trend was observed in the non-aromatic cultivars. Putrescine content was significantly reduced in the aromatic varieties compared to the non-aromatic ones which further assure its channelization toward aroma production.

The BADHs are responsible for the formation of glycine betaine using the substrate betaine aldehyde. Rice plants are considered as non-accumulators of glycine betaine (Srivong et al. 2008; Nakamura et al. 1997). Ghosh and Roychoudhury (2018) showed that the presence of glycine betaine in rice is independent of the aroma formation since both of them are dependent on the BADHs. Though the non-aromatic cultivars showed slight higher glycine betaine content, the difference between the aromatic and the non-aromatic cultivars with respect to the content of glycine betaine was negligible and thus can be inferred that the aroma-producing pathway is independent and not affected by the presence of glycine betaine.

5.1 Specificity of Rice BADH Enzymes Toward Different Substrates

The superfamily of aldehyde dehydrogenase (ALDH) are known as $NAD(P)^+$-dependent enzymes that help in metabolism of numerous significant biological intermediate aldehydes. ALDHs catalyze a restricted range of substrates and require either NAD^+ or $NAD(P)^+$ as cofactors. The categorization of the members of ALDH superfamily is determined by their aldehyde substrates like lactaldehyde, alcohol, and aminoaldehyde. Aminoaldehyde dehydrogenases are $NAD(P)^+$-dependent enzymes which are owned by the aldehyde dehydrogenase 9 (ALDH 9) family and are capable of catalyzing the oxidation of a wide range of aminoaldehydes to their respective ω-amino acids. Plant betaine aldehyde dehydrogenase helps in the oxidation of a variety of γ-aminoaldehydes along with the natural substrate betaine aldehyde. There are two homologs of rice (*Oryza sativa*), viz., OsBADH1 on chromosome 4 and OsBADH2 on chromosome 8, respectively, sharing 75% similarity in amino acid sequence. It has been hypothesized that *OSBADH1* is confined to the peroxisomes and *OsBADH2* is located in the cytoplasm instead of the nucleus. Both the BADH enzymes are capable of oxidizing C3 and C4 aminoaldehydes or medium-chain aldehydes like gamma-aminobutyraldehyde (GABald), 3-aminopropionaldehyde (AP-ald), 4-N-trimethylaminobutyraldehyde (TMAB-ald), and 3-N-trimethylaminopropionaldehyde (TMAP-ald) better than betaine aldehyde (betaine-ald) (Jiamsomboon et al. 2012).

Jiamsomboon et al. (2012) investigated the substrate specificity of both BADHs by inducing site-directed mutagenesis of amino acids that helps in catalysis and substrate recognition. Investigation about the binding of cofactor NAD^+ to both the enzymes revealed that the overall structure of the enzyme was unaffected due to the mutations at the active site of OsBADH, thus unaffecting drastically the conformational structure of the protein, or the way the cofactor would bind. The study revealed that binding affinity of BADH2 to NAD^+ is higher compared to BADH1. Enzyme kinetic experiments carried out in this study unveiled the fact that the enzymes catalyze the oxidation of GABald more efficiently than betaine-ald. OsBADH1 W172F and *Os*BADH2 W170F exhibited elevation in catalytic efficiency toward GABald. Experiments like molecular docking analysis and molecular dynamic stimulation were carried out to conclude the models for aldehyde substrate-bound complexes of OsBADHs. It was noted that the amino acid residues like E262, L263, C296, and W461 of OsBADH1 and E260, L261, C294, and W459 of OsBADH2, which were restricted within 5 Å of the OsBADH active site, were found to be interacting with GABald by forming hydrogen bonds in both the isomers. Residues like W163, N164, Q294, C296, and F397 of OsBADH1-betaine-ald and Y163, M167, W170, E260, S295, and C453 of OsBADH2-betaine-ald formed the main interactions. Hence, the study helped us in understanding how the rice BADHs identify aldehyde substrates differently. Bradbury et al. (2008) expressed cDNA clones of *BADH1* and *BADH2* in *E. coli*, and the enzymes were purified, followed by an analysis to study the affinity of the enzymes toward a number of substrates like betaine-ald, GABald, gamma-guanidinobutyraldehyde

(GGBald), and N-acetyl-γ-aminobutyraldehyde (NAGABald). The K_m values of the enzymes revealed that BADH2 showing optimum activity at pH 10 exhibited little or no affinity toward NAGABald while moderate affinity toward GGBald and betaine-ald. However, BADH1 activity, being optimum at pH 9.5, showed little or no activity toward betaine-ald and had moderate affinity toward GGBald, NAGABald, and GABald. Thus both the studies illuminate the substrate specificity of the rice BADHs through different techniques.

5.2 Effect of Salinity on BADHs

Fitzgerald et al. (2008) investigated the effect of salinity on the transcript levels of the genes coding for the formation of the enzymes, BADH1 and BADH2. Quantitative real-time PCR analysis revealed similar nature of both the genes in the developing seed stage, while increment in *BADH2* was significantly higher in both the leaves and mature seeds than *BADH1*. *BADH2* transcript level was higher in the non-aromatic cultivars at all the developmental stages under study. *BADH1* exhibited similar pattern of transcript levels in both the fragrant and non-fragrant cultivars. Both the *BADH1* transcript levels and the ratio of *BADH1/BADH2* transcripts were significantly elevated in the leaf tissue of aromatic and non-aromatic cultivars in response to salinity. However, no relationship could be derived between *BADH2* transcript levels and salt treatment, concluding the prominent role of *BADH1* and not *BADH2* in response to salinity stress.

5.3 Volatiles Imparting Aroma to the Fragrant Cultivars

The distinct flavor and robustness of fragrance in various aromatic rice cultivars are either associated with one prime volatile compound or several volatiles mixed together. Apart from 2-AP, other compounds, viz., alkanals, alk-2-enals, alka-2,4-dienals, 2-pentylfuran, and 2-phenylethanol, are known to be involved in the total fragrance profile of aromatic rice cultivars (Sakthivel et al. 2009). Sukhonthara et al. (2009) analyzed and compared the volatile oils from red and black rice bran using gas chromatography-mass spectrometry (GC-MS). Almost 129 components were estimated, among which 95% belonged to red rice bran and 95% belonged to black rice bran of the total oils represented. It was observed that the acid components were more in number in both the red and black bran of rice, followed by the aldehyde group. Myristic acid, nonanal, (E)-β-ocimene, and 6,10,14-trimethyl-2-pentadecanone served as the main volatile compounds in the red rice bran, whereas myristic acid, nonanal, caproic acid, pentadecanal, and pelargonic acid were the principal components in the black rice bran. Hexanal contents were found to be more elevated in the red rice bran compared to the black rice bran. The contents of nonanal, octanal, 2-methylnapthalene, (2E)-decenal, benzaldehyde, 2-heptanone, 1-octen-3-ol, (2E)-octenal, naphthalene, (2E)-nonenal, and (2E,4Z)-decadienal were registered to be higher in red rice bran compared to black rice bran. The

amount of n-pentanol was found to be elevated in the black rice bran compared to the red rice bran. Guaiacol has been registered as the principal component of black rice bran that contributes to the specific smoky or black rice-like aroma in cooked black rice. Guaiacol being the key odorant and contributor of smoky flavor to black rice bran is the main component that helps in distinguishing between red rice bran and black rice bran.

Liyanaarachchi et al. (2014) investigated the volatile components of brown rice samples of six Sri Lankan rice cultivars which included some traditional aromatic varieties as well. The study revealed that ketones, aldehydes, alcohols, and heterocyclic compounds comprised the volatiles present in the varieties. Most of the volatiles were present in the cultivars Lanka-Samurdhi and Suwandal, among the seven rice samples under study. The volatile profiling of Suwandal exhibited the presence of compounds like 2-butoxy ethanol, octanol, benzyl alcohol, hexanal, limonene, 1-pentanol, and 2-AP. Basmati showed the presence of the compounds, viz., indole, limonene, benzaldehyde, benzyl alcohol, and phenol. However, phenol was the only compound among the other volatiles that could be detected in the non-aromatic variety, Bg352. 2-AP, a heterocyclic compound that is considered as the key compound toward the contribution of aroma, was registered to be the highest in the cultivar Kuruluwee. Cultivars like Suwandal, Kuruluthuda, and Lanka-Samurdhi exhibited 4.5, 2.5, and 0.96% 2-AP contents, respectively; however, complete absence of 2-AP was noticed in non-aromatic Bg352. 2-AP was undetected in the Basmati variety that was procured from the supermarket. Among the detected aldehydes, the presence of hexanal was noticed only in Suwandal and Lanka-Samurdhi. Other alcohols like 2-butoxy ethanol, 1-pentanol, n-octanol, and benzyl alcohol were detected only in Suwandal. 2-Butoxy ethanol was undetected in all the other varieties. 1-Pentanol was found in Lanka-Samurdhi, while benzyl alcohol was also registered in Suwandal, Basmati, Kaluheenati, and Lanka-Samurdhi. The presence of octanol was registered in all the varieties. While Kuruluwee accounted for the highest content of octanol among all the other cultivars, Basmati exhibited total absence of octanol. Lanka-Samurdhi was registered to contain the highest amount of limonene, which is a terpene compound and 10.6% of the volatiles. Suwandal and Basmati exhibited 1.5 and 1.9% of limonene content.

6 Aromatic Rice and Abiotic Stress Response

6.1 Metal Stress

6.1.1 Cadmium (Cd)

Cadmium (Cd) is one of the most harmful and toxic heavy metals which is deposited in agricultural fields due to usage of certain phosphate fertilizers, metal smelting, sewage sludge containing cadmium, and other waste materials (ShekaKanu et al. 2017). Song et al. reported that cadmium can be taken up by rice which further causes morphological, physiological, and biochemical changes in rice seedlings. The visible effects of Cd toxicity in plants include growth retardation and chlorosis

leading to plant death. Cadmium consumption has dangerous effects on human health including cardiac failure, anemia, cancer, hypertension, emphysema, proteinuria, osteoporosis, etc. (ShekaKanu et al. 2017).

Majumdar et al. (2018) analyzed the harmful effects of Cd on seven non-aromatic (IR-64, Satabdi, Bandana, Swarna, Khanika, Palman, and Kariagora) and six aromatic (Badshabhog, Tulsibhog, Gobindobhog, Pusa Basmati, Tulaipanji, and Radhunipagal) rice varieties of West Bengal. The metabolites like free proline and malondialdehyde (MDA) content were found to be higher in the treated samples compared to the non-treated ones. The highest proline content was registered in Tulsibhog and the lowest in Badshabhog, while among the non-aromatic counterparts, the highest content was noted in Kariagora and lowest in IR-64. The MDA content was found to be higher in Tulsibhog and Radhunipagal among the aromatic rice cultivars, whereas Kariagora, Swarna, and Khanika registered the highest MDA content indicating higher tissue destruction. The photosynthetic pigment contents were also found to be lower in the treated samples compared to the untreated ones. This can be attributed to the fact that chlorophyll is degraded by chlorophyllase. Kariagora and Tulsibhog contained the least amount of photosynthetic pigments among the 13 cultivars. RAPD analysis showed that based on Gene-Targeted Sequencing (GTS) percentage, aromatic rice varieties held higher percentages compared to the non-aromatic ones. Badshabhog held the highest GTS percentage among the aromatic varieties, whereas Tulsibhog was found to contain the lowest. Among the non-aromatic ones, IR-64 showed the highest percentage, whereas Bandana accounted for the lowest one. Cluster analysis showed that IR-64 (among the non-aromatic varieties) and Tulsibhog (among the aromatic varieties) were placed in two isolated groups. Hence, the aromatic and non-aromatic cultivars were seen to behave in a similar way under Cd stress. The study helped in determining Cd-sensitive aromatic rice varieties like Tulsibhog (aromatic) and Cd-tolerant varieties like Badshabhog (aromatic).

6.2 Arsenic (As)

Arsenic has been enlisted as a chronic carcinogen and acute toxin when exposed at higher concentrations (IARC 2004). Arsenic is found in both organic and inorganic forms in the environment and can be affected by pH and redox conditions. Arsenate (As^{V}) and arsenite (As^{III}) (inorganic As forms) are mainly found in crop plants. While arsenate is observed in aerobic conditions, arsenite is found predominant in rice fields or submerged soil (Zhao et al. 2010; Sandhi et al. 2017). Toxic organic arsenic species like dimethylarsinic acid (DMA) and momomethylarsonic acid (MMA) are found in very low amounts in rice (Meharg et al. 2009; Sandhi et al. 2017). Exposure to higher amounts of inorganic As can lead to diseases such as various internal cancers and health problems such as skin cancer and diabetes (Guo et al. 1997). Bangladesh is well-known for its high As level in groundwater and its negative effects on the human health and environment (Smith et al. 2000).

Sandhi et al. (2017) studied the total As concentration difference among local aromatic rice (LAR) cultivar Kalijira and two high-yielding rice varieties (HYVs) (BRRI dhan 32 and BRRI dhan 28) in the paddy fields of Matlab region of Bangladesh which is considered as a hotspot for high inorganic As concentration in groundwater. The study registered that total As concentration was elevated in the de-husked grains of LAR compared to the husks, while it was totally reverse in case of HYVs. Almost two- to fivefold higher As concentration was registered in the soil samples in which LAR was grown than the ones which harbor HYVs. The accumulation factor of As was found to be lower in LAR than HYV. Thus, it was concluded that LAR was the safest among the rest for human consumption due to its low accumulating factor.

Al-Rmalli et al. (2012) conducted a study on Sylhet rice varieties using inductively coupled plasma mass spectroscopy (ICP-MS) to analyze the content of inorganic As and estimate the amounts of elements like cadmium, lead, manganese, zinc, and selenium. They revealed that non-aromatic rice from Sylhet contained the lowest amount of As as compared to the non-aromatic cultivars from other regions of Bangladesh. However, aromatic rice cultivars of Sylhet showed much lower As content compared to the non-aromatic cultivars of Sylhet. The aromatic rice cultivars also exhibited lower amount of harmful elements like cadmium and lead compared to the non-aromatic ones. Moreover, aromatic rice cultivars showed higher amount of essential elements like zinc and selenium compared to the non-aromatic counterparts. The study also stated that consuming the aromatic cultivars of Sylhet could lower the As intake by 40% compared to the non-aromatic ones. The study also revealed the fact that consumption of the aromatic cultivars of Sylhet could increase the uptake of essential elements like selenium and zinc by 46% and 23%, respectively. Islam et al. (2017) worked with large number of rice samples from 73 sub-districts in Bangladesh and determined the regional variation, distribution, and health risk associated with As. They reported that total As level was significantly lower in aromatic rice varieties (58 μg/kg) than the non-aromatic varieties (150 μg/kg) and it varied with the size of the grain.

6.3 Lead (Pb)

According to the new European REACH regulations, lead has been listed as "the chemical of great concern" since it has been recently reported as the second most harmful pollutant after As (Pourrut et al. 2011). Ashraf et al. (2017) studied the effects of different concentrations of Pb (400, 800, and 1200 ppm) against one control set (without Pb) in three aromatic rice cultivars, viz., Meixiangzhan-2, Xinagyaxiangzhan, and Basmati-385. The study registered reduction in the level of photosynthetic pigments (chlorophyll and carotenoids) and enhanced oxidative stress with the elevated production of hydrogen peroxide and MDA. The effects were more prominent in Xinagyaxiangzhan than the other cultivars. The production of protein, proline, and soluble sugars was being differentially affected owing to Pb stress. The production rates were noticeably higher in both the heading stage and

matured stages. Pb levels decreased the yield and yield components of the grains which further led to grain quality deterioration. The highest decrement was observed in Xinagyaxiangzhan (69.12%) followed by Meixiangzhan-2 (58.05%) and Basmati-385 (46.27%). Rice yields could be positively correlated with productive tillers/pots and grains per panicle, whereas it was negatively correlated with sterility percentage. The uptake of Pb from roots resulted in elevated levels of Pb in the plant parts in the following order: roots > stems > leaves > ears > grains. The accumulation of Pb in aboveground parts led to maximum losses in Xinagyaxiangzhan than the other two varieties. Basmati-385 was registered as the variety with least damages that might be owing to its defense system or less Pb accumulation in the aerial parts.

6.4 Salinity (NaCl) Stress

Most of the experiments conducted on salinity stress have ultimately subjected the crops to sudden salt shock, whereas the scenario is totally contrasting in the fields as the summer leads to gradual lowering of groundwater level. The method of salt acclimatization allows the crops to adapt gradually to stress and deal with the related injuries rather than typical salt shock experiments (Banerjee et al. 2019). Banerjee et al. (2019) investigated the role of certain metabolites like proline, P5C, methylglyoxal, GABA, glycine betaine, and polyamines which regulate the aroma formation pathway in fragrant rice cultivars as well as toward osmotolerance regulation pathway during environmental stresses. The study involved the acclimatization of four aromatic cultivars, viz., Gobindobhog, Kalonunia, Tulaipanji, and Radhunipagal, and one non-aromatic cultivar IR-64 toward gradually increasing NaCl concentration. The study revealed that the metabolites were channelized toward aroma production, except in the case of stressed seedlings of Kalonunia, where metabolites were more involved in stress amelioration. The results were further supported by the immunoblot analysis of BADH2, which elucidated the presence of elevated BADH2 protein in case of the stressed seedlings of Kalonunia. Roychoudhury et al. (2008) investigated the molecular and biochemical response of an aromatic rice cultivar Gobindobhog to 200 mM NaCl stress compared against salt-sensitive variety, M-1-48, and salt- tolerant variety Nonabokra. The maximum amount of damage was noticed in Gobindobhog after treatment with NaCl as noted by maximally increased root to shoot ratio, highest chlorophyll degradation, maximum foliar concentration of Na^+ ions, and peroxide content. The oxidative damage was further amplified due to putrescine accumulation and toxic products like malondialdehyde formed as a result of lipid peroxidation and enhanced lipoxygenase activity in both Gobindobhog and M-1-48. The highest activity of guaiacol peroxidase was observed in Gobindobhog, along with elevated levels of cysteine and proline responsible for stress management. The study revealed Gobindobhog as a highly salt-susceptible cultivar.

Hasan and Sarker (2013) studied the effect of NaCl (0, 0.2, 0.4, and 0.6%) on eight aromatic rice cultivars in the form of Parental, F_1, and F_2 generations on Murashige and Skoog media. Callus induction and plant regeneration was seen to

decrease with increasing amount of salt doses. In case of parental generation subjected to salt stress, the highest percentage of callus induction was shown by Jirabhog and Badshabhog (70%), while the lowest was registered in Kataribhog (25%). In case of plant regeneration under salinity, the parental generation of Uknimadhu (37.5%) showed the highest percentage, while the lowest was noted in Rajbhog (2.5%). In case of the F_1 generations subjected to salinity, the highest percentage of callus induction was noticed in Uknimadhu × Chinishakkhor (70%), and the lowest percentage was registered in Kalijira × BR 5 (5%). Plant regeneration was found to be highest in Uknimadhu × BR 5, while no regeneration was observed in Kalijira × Jirabhog. In case of the F_2 generations, Kalijira × Chinishakkhor (32.5%) showed the highest callus induction, while the lowest induction was exhibited by Jirabhog × BR 5 (5%). The highest percentage of plant regeneration was noticed in Uknimadhu × Kataribhog (10%), while the lowest percentage was exhibited by Jirabhog × BR 5, as there was no regeneration.

6.5 Drought

Yoshihashi et al. (2004) investigated that 2-AP content in aromatic cultivar Khao Dawk Mali 105 is dependent on drought stress. The variety Khao Dawk Mali 105 showed higher 2-AP content in the cropped rainfed paddy fields of northeastern region of Thailand. The Tungkularongkhai region was famous for producing highest-quality rice, showing the highest 2-AP content, while 2-AP content was found to be in lower amounts in the non-drought regions of Tungkularongkhai. Hence, it was concluded that dry climate influences elevated formation of 2-AP in Khao Dawk Mali 105.

Basu et al. (2010) investigated the effect of drought stress on three indica rice cultivars, viz., IR-29 (salt-sensitive), Pokkali (salt-tolerant), and aromatic rice Pusa Basmati to analyze the defense mechanism. A large amount of chlorophyll degradation and higher content of H_2O_2, malonaldehyde, and lipoxygenase levels were noticed in IR-29 and Pusa Basmati in comparison with Pokkali, thus indicating toward severe damages caused by water deficit. The reductions in the levels of antioxidants, mainly flavonoids and phenolics, were noted in IR-29 and Pusa Basmati. The activity of antioxidative enzymes like catalase and superoxide dismutase was registered to be lower in IR-29 and Pusa Basmati. Severe drought-influenced elevation of guaiacol peroxidase activity was noticed in IR-29 and Pusa Basmati. The study revealed that the aromatic rice cultivar Pusa Basmati behaved in a similar way to the salt-susceptible variety IR-29, thus proving high vulnerability to dehydration stress.

6.6 Cold

Cold stress is a much known problem among rice. Owing to the origin of rice in subtropical and tropical regions, rice behaves as a cold-sensitive crop, so that cold stress highly affects rice in reproductive stages resulting in serious yield losses and decreased production of grains. It also affects the vegetative stage by slowing down growth rate and lowering down the seedling vigor, thereby decreasing the number of seedlings, reducing tillering, and increasing plant mortality. It also results in panicle sterility. Cold stress affects stages like germination, booting, flowering, and grain filling (Ghadirnezhad and Fallah 2014).

Ghadirnezhad and Fallah (2014) evaluated the effect of cold stress in the flowering stages involving five varieties, viz., cultivars of shirudi, fajr, local tarom, hybrid, and line 843. The mentioned varieties were subjected to 13 °C (cold stress) and 32 °C (normal temperature). Flowering stage was chosen as the most prominent stage of study. After exposing the seedling to 13°°C for 15 days, it was noted that there is a significant decline in traits like number of panicles and number of full, empty, and total grains. Owing to the decrease in the mentioned characteristics, a significant decrease in yield was noted. According to the study, the most tolerant variety was found to be Shirudi in relation to the temperature, whereas the most sensitive variety in accordance with the temperature and maximum amount of recorded percentage decrease in yield was found to be local tarom variety.

6.7 Heat

Change in climate caused by emission of greenhouse gases and elevation of the atmospheric temperature affects agriculture. Presently, rice is grown at a temperature of 22 °C/28 °C, and additional changes in the temperature will affect the crop yield drastically. Excessive heat or heat stress causes irrevocable damage like retardation in plant growth, metabolic activities, pollen fertility, and seed setting which can result in huge reduction in rice yield. Heat stress is also known to accelerate reduction in the photosynthetic rate, leaf area, shoot grain mass, seed weight, and water use efficiency. Although high temperature is known to disrupt the vegetative and the reproductive stage, booting and flowering stage can prove to be the most critical ones leading to complete infertility in rice (Zafar et al. 2018).

Islam (2011) initiated a pot experiment by exposing five aromatic rice genotypes, viz., BRRI dhan 34, Uknimadhu, RM-100-16, KD_5 18–150, and Kalozira, to three temperatures, viz., ambient, 34 °C at booting stage, and 34 °C at grain filling stage for 7 days. The experiment revealed that photosynthetic rate, grain yield, and harvest index were decreased, while leaf conductance and transpiration rate increased with elevated temperature (34 °C) at both booting and grain filling stages. It was found that the total dry matter was the lowest at the booting stage when kept at 34 °C, while plant height and number of panicles per plant were unaffected by temperature treatments. BRRI dhan 34 exhibited the highest rate of photosynthesis, while RM-100-16 was registered with the highest conductance and transpiration rate.

Kd_5 18–150 exhibited higher grain yield, total dry matter per plant, and harvest index when subjected to temperature stress.

6.8 Shading Stress

The range of electromagnetic radiation in between 400 and 700 nm is known as the photosynthetically active radiation (PAR) which allowed the plants harness light for photosynthesis. Variation in PAR can have a serious effect on the growth and development of the plants. The intensity of solar radiation (SI) is a very important factor in photosynthesis since it determines the level of PAR obtained by any crop. Variation in solar radiation can affect crop yield due to the influence of factors like season, geographic location, cloud cover, atmospheric pollution, and water vapor content. Simultaneously, SI can also be affected by surrounding vegetation. Thus, shading stress is a considerable factor in environment occurring among plants beneath or among a canopy. Previous studies have shown that shading affects morphological and physiological characteristics as well as yield and quality of rice plants (Mo et al. 2015). Mo et al. (2015) investigated the impact of shading on GABA, proline, and 2-AP accumulation during grain filling period in two aromatic rice cultivars named 'Yuxiangyouzhan' and 'Nongxiang 18'. Their study revealed a significant increase in 2-AP and GABA contents in both the cultivars in all shading conditions; 2-AP contents were registered to be higher in the early grain filling stage when compared to the control (absence of shading). In Yuxiangyouzhan, a significant increase in the proline content from the whole grain filling stage to the early grain filling stage was noted, though no significant increase was noted in the late stage of grain filling. No significant impact on the proline content was noted in Nongxiang 18 at any shading treatment. Total nitrogen content also exhibited no significant changes between the shading treatments. Measurements of 11 volatiles, viz., (E)-2-hexanal, 1-hexanol, heptanal, 2-AP, octane, 1-heptanol, 1-octen-3-ol, octanal, benzyl alcohol, benzene acetaldehyde, and 3,8-dimethyl undecane, were carried out. In case of Yuxiangyouzhan, shading treatments significantly elevated the (E)-2-hexanal content. The latter grain filling stage exhibited the increment of 2-AP. Octane content was significantly lowered in both early and latter stage of grain filling. However, the whole grain filling stage registered the increment of octanal and benzyl alcohol. In case of Nongxiang 18, the early and the latter stages of grain filling registered an increase in the relative content of (E)-2-hexanal. While octane content was found to be decreased in the latter stage of grain filling, the same stage registered the increment for octanal. The content of benzyl alcohol was significantly increased in the whole grain filling and latter stage of grain filling. The whole grain filling stage and the early grain filling stage showed significant elevation in the level of 3,8-dimethyl undecane content. While drawing comparison between the two cultivars, it was noted that while Yuxiangyouzhan had significantly elevated levels of 2-AP content, Nongxiang 18 was registered to contain the most elevated levels of (E)-2-hexanal, heptanal, octane, and octanal contents.

Growth and yield were also affected in both the cultivars. All shading treatments led to a significant reduction in 1000-grain weight, grain yield, filled grain percentage, total dry weight, and harvest index in the cultivar Yuxiangyouzhan. A significant reduction in panicle number was also noted in the whole grain filling period and latter stage of grain filling in the abovementioned cultivar. In case of Nongxiang 18, a significant reduction in filled grain percentage and 1000-grain weight was noticed during whole grain filling stage and early grain filling, while a decrease in grain yield and total dry weight was observed during whole grain filling period. The grain quality was also affected due to all of the shading treatments. The shading treatment led to the increment in grain protein content in both the cultivars. Both the cultivars exhibited changes in characters related to the chalkiness trait. A significant reduction in the mean rate of the grain chalkiness was observed during whole grain filling and late grain filling. However, early grain filling period led to the significant increase in chalkiness.

6.9 Aging

Aging is known to enhance the cooking properties of rice. Aging method takes time and is also known to reduce desirable features including aroma of scented rice cultivars. Hence the technique accelerated aging (AA) was proposed to cut down the time of conventional aging. Study of accelerated aging in freshly harvested paddy with suitable grain moisture content by using wet or dry heat treatments was carried out, and it repeatedly improved cooking quality of rice similar to naturally aged rice. The impacts of AA on freshly harvested paddy were predicted as changes in aroma quality, volatile profile due to the diffusion of husk, and bran components into the endosperm of rice during moistening step along with relatively high temperature (Pisithkul et al. 2010). Pisithkul et al. (2010) analyzed the aroma quality and volatile components of freshly harvested milled rice of KMDL105 after exposing them to a treatment so as to detect any change that could have favorable or unfavorable effects on the aroma-producing volatiles. The study exhibited a reduction in 2-AP contents after AA treatment which revealed that the maximum reduction was in case of 100 °C for 100 min treatment (33.9%) and the minimum amount of reduction was noted in the treatment 120 °C for 25 min (21.8%) when compared with 2-AP content of freshly harvested milled rice. Thus, the result indicated toward the fact that 2-AP was greatly reduced in rice, when exposed for longer duration of AA treatment although the temperature applied for heating was lower (100 °C, 100 min). It was observed that AA treatments led to higher 2-AP accumulation in the treated samples than the naturally aged ones as the 2-AP contents reduced rapidly for storage of 3 months.

However, n-hexanal content in AA samples were noticeably decreased and were found to be lower than the freshly harvested milled rice samples. It was also reported that the n-hexanal amount was higher in paddy stored for 3 months which might be a result of degradation of lipid compounds of rice. Age acceleration at higher temperatures might have affected the lipid hydrolysis enzyme activity and

simultaneously promoted the release of this compound leading to the reduction of n-hexanal and hence enhancing aroma, as it is an off-odor compound in AA-treated samples. The study also investigated the volatile profiles of the samples. Thirteen compounds were identified, viz., n-hexanal, n-heptanal, 2-AP, benzaldehyde, 1-octen-3-ol, 2-pentylfuran, 1-octanol, n-nonanal, n-dodecane, n-decanal, n-tridecane, (E)-2-tetradecane, and n-tetradecane, in both freshly harvested and AA-treated samples. Both the freshly harvested samples and the aging-treated samples exhibited n-nonanal as the most abundant compound followed by benzaldehyde and n-hexanal. No additions of extra volatiles were observed in AA samples, but a reduction in the quantity of volatile components might have occurred as a result of oxidation of the rice constituents which leads to the release of highly volatile compounds from the rice kernel. However, aging treatment at 120 °C for 25 min exhibited the least amount of reduction of rice volatiles. Thus, the study concluded that rice treated with accelerated aging technique had better aroma quality compared to the 3-month naturally aged rice. Thus, freshly harvested seeds of KMDL105 can be subjected to accelerated aging technique while having the trait of high aroma content intact.

7 Developmental Stages Affecting Volatile Profiling in Rice

On the basis of developmental growth phases, rice can be classified into vegetative phase (germination to panicle emergence), reproductive phase (panicle emergence to heading), grain filling phase, and matured grain stage. In the following study, volatile profiling of scented cultivars involving seedling and tillering stages (vegetative phase), booting and flowering stages (reproductive phase), milky grain and dough grain stage (grain filling phase), and matured grain stage has been studied (Hinge et al. 2016). Hinge et al. (2016) performed the volatilome profiling of seven developing stages of two aromatic rice cultivars, Basmati-370 and Ambemohar-157, in comparison with one non-aromatic variety IR-64, using HS-SPME-GC-MS method. The study revealed the presence of 14 compounds including 2-AP that was specifically exhibited in the fragrant cultivars. Highest number of compounds was registered at the seedling stage due to the formation of maximum number of green leaf volatiles. The compounds were noticed to be decreasing gradually at the reproductive and maturity phases. 2-AP content was found to be the highest in the matured grains followed by grains at booting stage. The scented rice cultivars registered higher number of volatile compounds throughout all the seven developing stages compared to the non-aromatic cultivar. Among the 14 compounds exhibited specifically by the aromatic cultivars, eight compounds, viz., 2-AP (nutty or popcorn-like aroma), 2-acetyl-1H-pyrrole (musty and nutty aroma), β-ionone (raspberry, floral), (E,Z)-2,6-nonadienal (green, metallic), p-xylene (sweetish), methyl 2-aminobenzoate (sweet, grape fruity) with identifying odors, azulene, and acetic acid, 1,7,7-trimethylbicyclo(2,2,1)hept-2-yl ester without any distinctive odor, were listed among common volatiles in both the cultivars. Ghosh and Roychoudhury (2020) recently made a detailed study on the differential regulatory pattern of aroma-

associated genes in the developing stages of grains of four indigenous aromatic rice varieties, Gobindobhog, Radhunipagal, Kalonunia and Tulaipanji against the non-aromatic variety, IR-64.

7.1 Seedling Stage

The seedling stage registered the presence of 72 compounds in Basmati-370 and 70 compounds in Ambemohar-157 which contained volatiles from all the 13 classes, whereas IR-64 exhibited 58 compounds from 11 classes. The VOC classes mostly comprised of aliphatic aldehydes and alcohols. Apart from these, N-heterocyclic, aromatic hydrocarbons, and other compounds belonging to different classes were present. Amidst all these compounds, eight compounds were found in both the scented cultivars, viz., 2-AP, 2-acetyl-1H-pyrrole, (E)-5-methyl-4-decene, (E)-5-ethyl-6-methyl-3-hepten-2-one, p-xylene, 1-isopropyl-2-methylbenzene, 1-isopropyl-4-methylbenzene, and valencene. Ambemohar-157 exhibited the presence of seven specific compounds, viz., β-ionone, 2-hexyl-1-octanol, 2-hexadecanol, (E,Z)-2,6-nonadienal, 1H-indole, L-limonene, and acetic acid, 1,7,7-trimethylbicyclo (2,2,1)hept-2-yl ester. Basmati-370 exhibited the presence of seven specific compounds, viz., allylcyclohexane, (Z)-3-undecane, 6,10-dimethyl-2-undecanone, pinene, (Z)-2-heptanal, methyl-2-aminobenzoate, and 3,4-dimethylcyclohexanol.

7.2 Tillering Stage

Tillering stage registered the presence of 70 compounds in Basmati-370, 68 compounds in Ambemohar-157, and 58 compounds in IR-64 respectively. Among the fragrant cultivars, 13 classes of compounds were present, whereas the non-aromatic cultivar accounted for the presence of 12 classes. The aldehydes and alcohols accounted for 21–24% and 16–19% of the compounds, respectively. The presence of N-heterocyclic compounds (2-AP and 2-acetyl-1H-pyrrole) and 13 other compounds helped in comparative detection between the aromatic and non-aromatic cultivars. Eleven compounds were detected in both the fragrant cultivars. Compounds like camphene and acetic acid, 1,7,7-trimethylbicyclo(2,2,1)hept-2-yl ester were specifically found in Basmati-370, whereas 1H-indole and 3,7-dimethyloctanol were mainly noted in Ambemohar-157.

7.3 Booting Stage

The booting stage showed lowering of the number of compounds from 72–68 to 63–62 than the seedling and tillering stage. All compounds that were exhibited in this stage were reduced and owned by the chemical classes, viz., alkenes, alkanes, ketones, terpenes, aromatic hydrocarbons, and esters.

7.4 Flowering Stage

The flowering stage registered further reduction in the number of aromatic compounds. Basmati-370, Ambemohar-157, and IR-64 exhibited the presence of 55, 51, and 48 compounds, respectively. Reduction in the classes of compounds like alkenes, esters, terpenes, alcohols, aldehydes, and phenols was observed when compared to the seedling and tillering stage. Total absence of aromatic hydrocarbons was noted in the stage.

7.5 Milky Grain Stage

In the milky grain stage, the number of compounds was reduced to 58, 56, and 52 in Basmati-370, Ambemohar-157, and IR-64, respectively. While 12 classes of compounds were detected in the fragrant varieties, the non-fragrant cultivar accounted for compounds belonging to ten classes only. At this stage, total absence of carboxylic acid was noted. Maximum numbers of compounds were present from the terpenes in all the three cultivars.

7.6 Dough Grain Stage

The dough grain stage registered the presence of 52 compounds in Ambemohar-157, 53 compounds in Basmati-370, and 50 compounds in IR-64. While 11 classes of compounds were detected in the fragrant varieties, the non-fragrant cultivar accounted for compounds belonging to ten classes only. The presence of N-heterocyclic compounds was noted only in the fragrant cultivars. Complete absence of aromatic hydrocarbons and carboxylic acid was noticed in this stage. The number and percentage of terpenes were further reduced in this stage compared to first four stages.

7.7 Mature Grain Stage

In the mature grain stage, 51 compounds from 12 chemical classes in Ambemohar-157 and 54 compounds from 11 chemical classes were noticed in Basmati-370. Compounds belonging to chemical classes like N-heterocyclic compounds, terpenes, aromatic hydrocarbons, alcohols, ketones, alkanes, alkenes, aliphatic hydrocarbons, and phenols were present. The major class that helped in distinguishing the fragrant cultivars from the non-fragrant ones were the N-heterocyclic compounds. Among all the volatiles detected in the profiling study, aldehydes, alcohols, and phenols accounted for almost 40% of the volatiles across all the developmental stages in all the cultivars. N-Heterocyclic compounds followed by aldehydes were the two most important groups that were involved in imparting aroma to the rice varieties. Among all the identified aldehydes, ten aliphatic aldehydes, viz., pentanal, heptanal,

(Z)-2-heptenal, (E,E)-2,4-decadienal, (E)-2-octenal, nonanal, (E,Z)-2,6-nonadienal, decanal, (E,E)-2,4-nonadienal, and β-cyclocitral, and three aromatic aldehydes, viz., benzaldehyde, phenylacetaldehyde, and vanillin, were among the important and mandatory volatiles that imparted the characteristic odor and flavor to fragrant rice. Thus, the study helped in deciphering the fact that with changing developmental stages, the volatile profiling of rice changes.

8 Effect of Growth Regulators on Aroma Content and Volatiles

Plant growth regulators are very significant in agriculture since that could lead to the increment of crop yield at an unparallel rate and would also remove genetic and environmental hindrance in crop production. The efficiency of plant growth regulators relies on several factors, viz., methods, time of application, concentration, etc. Different modes of application of the plant growth regulators, viz., seed soaking, foliar spray, stem injection, and soil application, are known to alter the plant physiology differently. Thus, the plant growth regulators play important role in the physiological processes and other important functions in the plant (Kumar et al. 2018).

Goufo et al. (2011) have elucidated the impact of some plant growth regulators on the volatile nature of aroma in two rice cultivars, viz., Guixiangzhan and Peizaruanxiang. The different treatments included growth regulators like gibberellic acid, indole-3-acetic acid (IAA), and paclobutrazol and a regulator mixture comprising paclobutrazol, proline, and zinc chloride. Twelve odor active compounds could be extracted using headspace coupled with gas chromatography. An olfactory evaluation exhibited higher intensity of aroma in control samples than the ones treated with growth regulators, and the difference in the aroma was related to the concentration of 2-AP and lipid oxidation volatiles. In case of Guixiangzhan, the early season registered that the least amount of reduction of 2-AP occurred in the samples treated with IAA (2.05 ng/g), followed by paclobutrazol (1.76 ng/g), regulator mixture (1.63 ng/g), and gibberellic acid (1.46 ng/g) when compared with the control (3.6 ng/g). During the late season, the least amount of 2-AP reduction was noted during treatment with paclobutrazol (4.05 ng/g) followed by IAA (3.46 ng/g), gibberellic acid (2.37 ng/g), and regulator mixture (1.9 ng/g) when compared with the control samples (6.52 ng/g). In case of the variety Peizaruanxiang, the early season registered the least reduction of 2-AP during treatment with gibberellic acid (0.75 ng/g), followed by IAA (0.74 ng/g), regulator mixture (0.59 ng/g), and paclobutrazol (0.51 ng/g) in comparison with the control (1.15 ng/g). However, the late season registered the minimum amount of 2-AP reduction during treatment with IAA (1.36 ng/g), followed by paclobutrazol (1.2 ng/g), gibberellic acid (1.17 ng/g), and regulator mixture (0.9 ng/g) in comparison with control (1.71 ng/g) samples.

A significant decrease was noted in the lipid-derived volatiles in the two rice samples as indicated by the reduction in most of the aldehydes and alcohols.

Nonanal was the most important alcohol found in the two cultivars and maintained its concentration in highest amount throughout the investigation and thus can be considered in having a large impact on the overall aroma of these two varieties. It was found hexanal had the biggest contribution in the odor of the milled rice during early storage, contradictory to octanal which exhibited its influence during the long-term storage. (E)-2-Hexanal decreased in the early season of Guixiangzhan and Peizaruanxiang, whereas Peizaruanxiang exhibited almost constant concentration for the samples treated with IAA, paclobutrazol, and regulator mixture during the late season. Important lipid-derived alcohols like 1-hexanol and 1-pentanol which are known for their role in distinguishing between aromatic and non-aromatic cultivars were observed to be decreasing in the early and late seasons of both the cultivars under the effect of different treatments. Volatiles that are often recognized in rice are (E)-2-octenal, 2-pentylfuran, and (E, E)-2,4-decadienal which were noticeably reduced as a result of foliar spray of the mentioned treatments. Inhibition of lipid oxidation was confirmed by the reduction in the amount of a non-volatile product of lipid peroxidation known as malonaldehyde, in the samples under treatment. Unlike the other volatiles which increased their concentration with the storage time in the control samples, 1-octanol, 1-nonanol, and benzaldehyde in Guixiangzhan and 1-nonanol, (E)-2-hexanal, and decanal in Peizaruanxiang registered higher concentration in the earlier season than the late season.

Benzaldehyde is the aromatic compound with almond-like odorant and helps in discriminating between aromatic and non-aromatic cultivars. The compound was detected only in the control and samples treated with IAA during the late season. Thus, it can be said that foliar spray of growth regulators decreased the amount of benzaldehyde.

Improvement of the plants after application of the growth regulators was observed in one or more treatments. During both seasons, samples treated with gibberellic acid exhibited the highest grain yield in Guixiangzhan and Peizaruanxiang. Samples treated with paclobutrazol produced the highest head rice rate along with highest grain vitreosity in Peizaruanxiang. However, treating rice with IAA was found to be beneficial in terms of 1000-grain weight, head rice yield, grain vitreosity, amylose content, and protein content. Thus the study concluded that the application of growth regulators at panicle emergence helps in the improvement of the grain yield and quality, but a significant reduction in aroma was also noticed. So, it is suggested that the application of growth regulators on rice plants becomes mandatory owing to reasons like improved yield and quality and stress amelioration. However, the process should be carried out with careful monitoring of the aroma level in rice plants.

8.1 Abscisic Acid Effect on Fragrant Variety

Kong and Zhao (2014a) studied the effects of abscisic acid (ABA) on the volatile components producing aroma in the indigenous aromatic Kam sweet rice variety named Gou Cengao using GC-O and SPME-GC-MS. Among the different volatiles,

2-AP and nonanal were found in prominent amounts, while the latter was registered to have the highest odor active value (OAV) in the grains, which decreased considerably when ABA was applied in rice seedlings. Lipoxygenase 3 of rice (*OsLOX3*) showed elevated expression in the aromatic variety, Gou Cengao, when compared against a non-aromatic cultivar Lailong after pollination. Lipoxygenase activity was also registered to be higher in the aromatic variety than the non-aromatic one. Hence, a negative Pearson correlation was derived between the concentration of exogenous ABA applied and relative expression of rice *LOX3*. This suggested an inhibitory impact of ABA on the nonanal biosynthetic pathway, thereby restricting aroma content.

Roychoudhury et al. (2009) studied the manner in which different rice genotypes would differ with respect to the gene expression pattern of two ABA-inducible genes, viz., *Rab16A* and *SamDC*, in the dry, water-imbibed, and ABA-imbibed seeds of Nonabokra (salt-tolerant), M-1-48 (salt-sensitive), and Gobindobhog (aromatic). The results depicted that M-1-48 and Gobindobhog exhibited extremely low and almost undetectable gene transcript levels for *Rab16A* and *SamDC* in the dry and water-imbibed conditions, whereas both the transcripts showed higher expression in the ABA-imbibed seeds of the above cultivars. The protein expression of RAB16A and SAMDC was also similar to the expression of the transcript levels. Thus the study revealed similar behavior of salt-sensitive M-1-48 and aromatic rice Gobindobhog when exposed to exogenous ABA.

8.2 Effect of Jasmonic Acid on Fragrant Variety

Kong and Zhao (2014b) investigated the effects of jasmonic acid on the volatile nature of aroma of the variety Kam sweet rice Gou Cengao and non-aromatic cultivar Lailong using GC-O and GC-MS. The highest amount among all the odor active values (OAVs) after application of jasmonic acid was displayed by nonanal followed by 2-AP. *OsLOX3* gene showed elevated expression in the aromatic cultivars which could be correlated with the LOX activity found to be higher in the fragrant variety compared to the non-fragrant one. Thus, a positive correlation between endogenous jasmonic acid and LOX was derived. Similar correlation was drawn between the concentrations of nonanal and LOX activity. Thus, the study elucidated the fact that jasmonic acid increases the aroma content by positively influencing nonanal biosynthetic pathway. Jasmonic acid enhances the aroma content, while ABA decreases the same, deciphering the fact that different hormonal treatments have different effect on the aroma formation of the aromatic cultivars.

9 Diverse Factors Affecting Volatile Formation in Fragrant Varieties

There are several factors which can improve the quality of the aroma in the fragrant cultivars.

9.1 Role of Nitrogen and Irrigation in Enhancement of Aroma

Nitrogen and water are known to play important roles in the growth and development of aromatic rice cultivars. Asian countries are known for their restricted rice production owing to the shortage of water prevailing there. Hence, irrigation can be considered as an important factor influencing rice production (Ren et al. 2017). Ren et al. (2017) investigated the effects of combination of water and nitrogen at tillering, booting, and grain filling stages of two fragrant cultivars, Yungao and Yundi. The first treatment known as the conventional practice involved the usage of 30 kg/hm^2 of nitrogen at the three stages along with 0 kPa of water in both the early- and late-season rice. The second treatment named as treatment management practice (TNW) involved the application of 60 kg/hm^2 of nitrogen with heavy drought condition. The study revealed elevation in the panicle number, seed setting rate, 1000-grain weight, and grain yield for both the cultivars in the early and late season in case of the TNW treatment. 2-AP content was found to be elevated in both the cultivars during both the seasons at the grain filling stage. Significant increment in the 2-AP content in the grains of Yungao was noticed at maturity in 7 days after anthesis and 14 days after anthesis in the early and late season, respectively. However, in Yundi, significant increment in 2-AP content was noticed in 7, 14, and 21 days after anthesis in the early season and 14 days after anthesis in the late season. Grain filling stage showed a reduction in 2-AP content in the leaves; however, proline content was elevated in the same stage.

9.2 Gamma Irradiation Result in Increased Aroma in the Fragrant Cultivars

Gamma radiation is a type of ionizing radiation that induces mutation in different organisms. Gamma irradiation helps in improving grain quality, plant resistance toward certain diseases and stresses, effect on germination, and variation of root and shoot length. Previous studies have stated changes in certain compounds like increasing the pasting values of rice while decreasing the free fatty acids of rice grains along with reduction in odor scores in gamma irradiated brown rice (Sansenya et al. 2017). The effects of gamma irradiation on 2-AP and GABA content and volatile compounds of germinated Thai upland rice were studied. Almost 23-fold higher 2-AP content in the cultivar (germinated within 24 h) was noted at 20 Gy gamma doses. A reduction in GABA content upon increasing the gamma doses was also noticed, particularly at 300 Gy where a sharp 2.6-fold decrease in GABA content was noticed in comparison with the non-irradiated rice. Reduction in the volatile profiles was also noted with increasing doses of gamma rays. However, few volatiles showed their presence at 60, 80, 100, and 300 Gy doses of gamma radiation. Octanal was most prominent among all the other volatiles and was found to be stronger in the irradiated rice when compared to the non-irradiated ones. Thus, the study derived a stable relationship between aroma formation and gamma irradiation.

9.3 Application of 2-AP, Zinc, and Lanthanum Influences 2-AP Contents

Important elements like zinc (Zn) and lanthanum (La) are known to affect the aroma production in fragrant cultivars. Exogenous application of these elements in the form of either base fertilizer or foliar spray has improved 2-AP contents in scented rice varieties (Mo et al. 2016). Mo et al. (2016) elucidated the effects of 2-AP, Zn, and La on 2-AP levels of three fragrant cultivars, viz., Guixiangzhan, Pin 14, and Pin 15. The investigation showed significant elevation in the concentration of proline when the culture medium was augmented with 2-AP, Zn, and La. A similar increase of 2-AP along with an elevation of PDH activity was noticed among the grains. Thus, a positive correlation was deduced between 2-AP concentration, proline content, and PDH activity in grains and 2-AP in culture medium. The study also predicted possible roles of Zn and La in the formation of aroma as their presence leads to increment of proline, PDH activities, and 2-AP contents.

9.4 Manganese Helps in Inducing Aroma

Aroma in scented cultivars can be enhanced by nutrients in low levels. Manganese (Mn) is considered as a significant nutrient in plant system owing to its important role as a cofactor for enzymes that are necessary for different metabolic pathways. Mn-deprived soils drastically affect plant growth and quality. Fertilizers comprising Mn have proven to be beneficial for crops in terms of yield and quality by upgrading soil status and efficiency of photosynthesis (Li et al. 2016).

Li et al. (2016) investigated the role of Mn in aroma formation owing to its diverse roles in plant physiological processes and metabolic pathways. The study involved the application of $MnSO_4$ in four different concentrations, viz., 100, 150, 200, and 250 mg per pot, in two aromatic cultivars, viz., Meixiangzhan and Nongxiang 18. The study revealed a positive impact of Mn on growth- and yield-related features along with some quality attributes in rice grains. The investigation exhibited elevated levels of proline, Δ^1-pyrroline-5-carboxylate (P5C), and soluble proteins. The analysis also showed higher activities of significant enzymes playing important role in the metabolic pathway of aroma formation, viz., OAT, P5CS, and PDH, which leads to further increment in 2-AP contents in rice grains. The 2-AP content in Nongxiang 18 was found to be higher than Meixiangzhan in both the early and late seasons.

9.5 Rhizobacterial Inoculation Helps in Increment of 2-AP in Basmati Rice

Plant growth-promoting rhizobacteria (PGPR) play an essential role in the production of secondary metabolites by host plants. The biological approaches like the plant-microbe interaction in the rhizosphere help in the growth and development of

plants and are becoming more desirable in the era of sustainable crop production (Deshmukh et al. 2016). Deshmukh et al. (2016) investigated the effects of rhizobacteria from soils cultivated with Basmati and non-Basmati rice for a long time span on 2-AP concentrations of Basmati rice. Bacterial isolates from Basmati origin exhibited higher 2-AP contents when compared to the non-Basmati control. It was observed that strains of *Acinetobacter* sp. from the Basmati origin exhibited greater potential of 2-AP production than the other rhizobacterial isolates. The analysis chose five strains of bacteria comprising *Acinetobacter* sp. and *Enterobacter ludwugii* having different potency of 2-AP production and used in the inoculation of the root system of Basmati-370. The analysis revealed an elevation in 2-AP content. The impact of inoculation was more prominent with the isolates having high 2-AP-producing capacity.

9.6 Conditions of Pre-harvest Treatment

Goufo et al. (2010) investigated different strategies affecting 2-AP content by subjecting two cultivars, viz., Guixiangzhan and Peizaruanxiang, to two pre-harvest treatments, viz., planting density and harvest date with different storage conditions (3–6 months) and at variable temperature, viz., −4 °C, 8 °C and 30 °C. The study revealed elevation in the concentration of 2-AP in Guixiangzhan and Peizaruanxiang with lower planting density, early harvesting at 10 days after heading, and shortest storage time of 3 months while maintaining temperature as low as −4 °C. Thus the manipulation to increase the aroma was achieved by using the mentioned treatments, and this method could be used in future as a technique to maintain and enhance the aroma of fragrant cultivars.

9.7 Increment of 2-AP and Aroma-Related Compounds due to the Practice of Alternate Wetting and Drying (AWD) Method

AWD is a powerful and efficient technique that helps in water saving by improving and restricting its use. The technique has positive impact on rice growth, development, yield, and attributes related to grain quality (Bao et al. 2018). Ren et al. (2017) reported the positive impact of AWD in enhancement of 2-AP at the tillering, booting, and grain filling stages. Bao et al. (2018) applied three irrigation techniques, viz., conventional irrigation (CI), alternate wetting and moderate drying (WMD), and alternate wetting and sever drying (WSD), on two scented cultivars, viz., Meixiangzhan 2 and Xiangyaxiangzhan. The study revealed elevation in 2-AP contents in both the cultivars under WMD and WSD treatments than CI. Important intermediate precursors of 2-AP, viz., Δ^1-pyrroline and P5C, were found to be higher in both the cultivars under WMD and WSD. GABA levels were observed to decrease under the AWD treatments. Important enzymes involved in the metabolic pathway of aroma formation, viz., PDH, P5CS, DAO, and BADH2, were

differentially influenced by AWD treatments. Elevation in PDH and DAO activities was noticed in both the cultivars under the AWD treatments. P5CS activity was elevated only in WSD treatments when compared with the CI in both the cultivars. No significant difference was found between CI and AWD treatments with respect to the OAT activities. BADH2 activity was reduced significantly under WSD in both the cultivars, whereas WMD was registered to behave statistically similar to the CI treatments. Real-time PCR revealed that the expression of genes, viz., *PDH*, *P5CS*, and *DAO*, was elevated by AWD treatments. *BADH2* expression levels were reduced in the AWD treatments for both the cultivars. Thus, the result clearly depicts a positive influence of the AWD treatments on the aroma-producing pathway.

9.8 Comparison of Environmental Effect of Greenhouse and Open Air Condition on Aroma of Fragrant Cultivars

Environmental factors play an important role in influencing the quality of aroma and yield of the scented rice cultivars. The aroma of fragrant rice is an outcome of genetic as well as environmental determinants. Thus, aroma flavor can be better regulated by good management practices ruled by different environmental factors during growth (Boontakham et al. 2019). Boontakham et al. (2019) investigated the effects of greenhouse and open air condition (difference of 6 °C) on the aroma quality and production yield of one Jasmine rice Khao Dawk Mali 105 (photoperiod-sensitive cultivar) and Pathum Thani 1 (PTT1) (photoperiod-insensitive cultivar). The factors like soil type (clay loam and sandy loam) and water stress were also applied. The study revealed that 2-AP level for leaves of both the cultivars was different depending upon the soil type. Khao Dawk Mali 105 exhibited higher 2-AP content in clay loam soil than PTT1. However, the pattern was found to be opposite in sandy loam soil. The plants grown under greenhouse condition exhibited lower 2-AP content as well as registered reduction in grain yield level. Though water stress was observed to increase the 2-AP content, it decreased the grain yield for all the growing conditions. The study helped in understanding the most significant impact on the 2-AP contents by temperature, while grain yield was influenced more by the soil type.

10 Techniques Leading to Enhancement of Aroma in Rice

Technologies focussing primarily on genome manipulation have evolved in the recent times and have created a true revolution in the field of genetic engineering and biotechnology. The aim of such techniques is to produce plants with important compositional properties that could enhance a particular trait like resistance toward biotic and abiotic stresses. Several molecular and genetic mechanisms have been introduced in recent times, viz., zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) (Shah et al. 2018). These novel technologies

are created with the aim of creating DNA double-stranded breaks in diverse model and crop plants (Shan et al. 2015).

10.1 TALEN Technology-Mediated Knockout of *OsBADH2* Gene

Transcription activator-like effector (TALE) proteins were identified with the aim to detect and trigger specific plant promoter with the help of a set of tandem repeats that gave rise to the creation of a novel genome editing system that involved the use of chimeric nucleases, hence termed as TALE nucleases (TALENs). TALE proteins are composed of a central domain which helps to bind DNA, nuclear localization signal, and a domain that acts as an activator of transcription of the target gene (Shah et al. 2018). Shan et al. (2015) engineered TALENs to distort the *BADH2* gene. Sangers sequencing confirmed indel (insertion-deletion) mutations at the target site of TALEN. Among the 20 transgenic hygromycin-resistant lines, six heterogeneous mutants were retrieved. It was found that all the six mutants transmitted *BADH2* mutations to T_1 generation. Mutant plants bearing the desired gene without the TALEN transgene were acquired during the segregation of T_1 and T_2 generations. The 2-AP levels of the T_1 generation containing homozygous mutations exhibited an elevation from 0 to 0.35–0.75 mg/kg, which was similar to the 2-AP content of the positive control variety, thus proving that the TALEN technology is useful for introducing agronomic traits like aroma in the non-aromatic cultivars of rice.

10.2 Transgenic Rice Leading to Downregulation of OsBADH2 Using RNA Interference (RNAi) Enhancing Aroma

Plants, which have their DNA modified using several techniques of genetic engineering with the ambition of introducing a new trait in a plant which does not occur originally in the species, are termed as transgenic plants. The concept of transgenics arrived with the aim to achieve advantages like improving shelf life, higher yield, improved quality, resistance toward pests, and tolerance toward heat, cold, drought, and various other biotic and abiotic stresses (Rani and Usha 2013).

The technique which involves the limiting of transcript level either by activation of sequence-specific RNA degradation process (posttranscriptional gene silencing) or by suppression of the important method of transcription (transcriptional gene silencing) is known as RNA silencing which has been reportedly considered as a novel gene regulation (Agarwal et al. 2003). Niu et al. (2008) sequenced the *OsBADH2* locus of 13 fragrant and 6 non-fragrant rice accessions that are the cultivars grown across parts of China. Multiple mutations identical to the *fgr* allele were studied in the Chinese fragrant cultivars. The following investigation involved the technique of RNA interference combined with *Agrobacterium tumefaciens*-mediated T-DNA transfer to generate a huge number of transgenic rice plants. These plants expressed the *OsBADH2-RNAi* by introducing the construct in non-fragrant rice cv. Nipponbare. Among 97 *hpt* (*hygromycin*

phosphotransferase)-positive plants, 21 exhibited abundant fragrance emission. Ten plants from T_0 generation were selected for the propagation of T_1 generation plants. A correlation between *OsBADH2-RNAi* transgene integration and apparent aroma production by sensory evaluation was noticed in all the ten T_1 generation plants. GC-MS confirmed the fact that significant production of the volatile 2-AP was detected in both Thai Hom Mali 105 and transgenic line (*OsB2-Rc*) which was undetectable in the wild-type control. GC-MS analysis was carried out in T_2 mature plants from five *OsBADH2-RNAi* and four non-fragrant wild-type cultivars segregated out of the transgenic line *OsB2-Rc*, which revealed 2-AP accumulation in all the downregulated plants while undetectable 2-AP in the wild-type cultivars. Correlated results were obtained for endogenous *BADH2* transcript levels in *OsBADH2-RNAi* repression line which confirmed lower transcript levels for the *OsBADH2-RNAi* line when compared with the wild-type plants. *OsBADH1* transcript levels were also measured for the *OsBADH2-RNAi* repression lines which revealed that *OsBADH1* remained unaffected with the downregulation of *OsBADH2* lines. Hence, the downregulation of *OsBADH2* was seen to elevate the 2-AP levels and lowered *OsBADH2* transcript levels in the transgenic line, while *OsBADH1* remained unaffected.

10.3 Overexpression of the *P5CS* Gene to Enhance Aroma in the Aromatic Rice Cultivars

P5CS gene is the key enzyme of proline synthesis, and proline is one of the main precursor amino acids in the 2-AP biosynthesis. The hypothesis of overexpression of the *P5CS* gene in fragrant cultivars might therefore lead to increasing amount of 2-AP (Kaikavoosi et al. 2015). Kaikavoosi et al. (2015) used two scented cultivars Ambemohar-157 and Indrayani to overexpress *P5CS* gene using *Agrobacterium tumefaciens*-mediated transformation. Overexpression of *P5CS* gene leads to elevation in the level of proline content; increment in the P5CS enzyme activity alone caused enhancement of 2-AP content to almost twofold in both the cultivars.

10.4 Significant Mutations in the BADH2 Protein Leading to Enhancement of Fragrance in Rice Cultivars

Mutated BADH2 is the main enzyme responsible for the formation of 2-AP in fragrant cultivars. In the fragrant cultivars, truncation in *BADH2* gene results in the accumulation of GABald, leading to the formation of 2-AP (Kamaraj and Purohit 2013). Rice *BADH2* is a member of the aldehyde dehydrogenase (ALDH) superfamily which consists of different enzymes that are known to catalyze the irreversible $NAD(P)^+$-dependent oxidation of a broad range of aliphatic and aromatic aldehydes to their respective carboxylic acid (Niu et al. 2008). Various studies have illuminated the significance of active site residues in the ALDH superfamily. The mutation which involves the catalytic site C294 to alanine leads to total loss of

enzyme activity. The mutation at E260 to alanine leads to the abolishing of the enzyme activity. However, the mutation at N162 causes reduction in affinity of the enzyme toward the substrate (Kamaraj and Purohit 2013). Kamaraj and Purohit (2013) investigated the interactive behavior of model structures of wild-type and mutant BADH2 enzyme and substrate GABald using in silico approach. Quantitative structural evaluations and salt bridge analysis helped to study the stability of BADH2 enzyme after mutation. The investigation revealed that the mutant forms helped in determining the decisive catalytic efficiency of the enzyme toward the substrate GABald. The mutant forms of the enzyme BADH2 did not allow it to interact properly with the substrate, which further led to the accumulation of the substrate and hence resulted in the formation of 2-AP. Quantitative and docking experiments revealed that $BADH2^{N162A}$ was considerably the most fragrant form of mutants when compared with the normal BADH2 and the two other mutants. The order of fragrance in rice as depicted by the mutants is as follows: $BADH2 < BADH2^{C294A} < BADH2^{E260A} < BADH2^{N162A}$. This proved that mutations in BADH2 protein can enhance the fragrance in rice.

11 Conclusion and Future Perspectives

India has a vast storehouse of several indigenous aromatic rice varieties, much of which has been largely unexplored. A thorough investigation of such varieties, in terms of the different regulators and external factors affecting aroma level, is essential in order to understand the mechanism of aroma formation in such varieties so as to ensure popularization of these varieties in the international market, thereby stimulating their production, global demand, and export.

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Genomics and Genetic Engineering of Rice Elucidating Cross Talk Between Stress Signaling and Nutrition Enhancement via Regulation of Antioxidant, Osmolyte, and Metabolite Levels

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Abstract

Rice (*Oryza sativa* L.) is the main source of staple food for human population. Salinity is the major problem for agricultural production, and it affects rice production globally. Salinity induces the production of reactive oxygen species (ROS) in plant cells. ROS can act as signaling molecules, mediating many key physiological processes. Osmotic adjustment has been shown to be an effective component of stress tolerance, and accumulation of osmoprotectants is a common response observed in different plant systems. Information on the metabolic pathways of these compatible solutes for their regulation, enzymes involved, and compartmentalization are well characterized in most important plant species. Different approaches have been developed and exploited to ameliorate the harmful effects of salinity on rice production. Genomics approaches have the potential to accelerate breeding process for the development of salt-tolerant crop cultivars. Identified genomic regions associated with salinity tolerance accelerated molecular breeding efforts to develop salt-tolerant rice cultivars. Development of genetically engineered plants with enhanced tolerance to salinity is an important challenge in rice biotechnology research. Genetic engineering has been used as a prominent tool for rice improvement. In this chapter, a detailed description of molecular mapping techniques, and major findings made by these techniques, is presented. We discuss the mechanisms by which rice perceive environmental signals and transmit them to cellular machinery to activate adaptive responses and to impart stress tolerance. Finally, we provide an overview of recent developments on the production of various transgenic lines in rice that are highly promising for stress tolerance.

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Antioxidant · Genomics, genetic engineering · Osmolyte · Rice · Stress signaling

1 Introduction

Rice (*Oryza sativa* L.) belongs to family Poaceae and genus *Oryza*. Its genome size is approximately 430 Mb contained in 12 chromosomes. Rice is widely grown as a major staple food crop for the world's human population. A significant yield loss in this crop is due to various biotic and abiotic stresses including insect pests, diseases, drought, salinity, adverse temperature, and submergence. Development of abiotic stress-tolerant rice genotypes with high grain yield is the major objective now in rice breeding and genomic research. Salinity has been a key abiotic constraint devastating crop production worldwide. Attempts in understanding salt tolerance mechanisms has revealed several key enzymes and altered biochemical pathways inferring resistance to crop plants against salt stress. An understanding of the specific response of rice to ion accumulation at the toxic level can aid in identifying the key factors responsible for retarded growth and limited production of rice with the future scope of mitigating the same. To combat stress, plant metabolism is altered in many different ways including compatible solute production to stabilize proteins and cellular structures and/or to maintain cell turgor by osmotic adjustment and redox metabolism to remove excess levels of ROS and re-establish the cellular redox balance (Chinnusamy and Zhu 2009; Janská et al. 2010; Krasensky and Jonak 2012). Osmotic adjustment in mediating stress tolerance and protecting subcellular structure has been considered as a central dogma in stress physiology (Hare et al. 1998); however, it is still debated whether increased osmolyte accumulation can benefit crop yield (Serraj and Sinclair 2002). Signaling molecules (hormones, growth regulators, proteins, amino acids, nucleotides, etc.) are essential for the growth, development, and adaptation of plants, as well as for the activation of their antioxidant responses to a number of environmental stress factors such as extreme temperatures, light, drought, salinity, heavy metals, herbicides, pathogens, and others (Gururani et al. 2015; Dmitriev 2003). Since large portions of rice-growing areas are affected by abiotic stress conditions, it would be difficult to meet the future food demands of ever-increasing world population (Hayashi et al. 1997). Efforts involving conventional breeding methods for improving traits that confer tolerance to the abiotic stresses have met with limited success (Sakamoto and Murata 2000). Therefore, to meet the food demands of the growing world population, conventional breeding methods need to be combined with tools such as molecular markers and genomics. Several biotechnological approaches are adopted to increase quality and quantity of rice as well as its resistance to pests, diseases, and environmental stresses (Prasad et al. 2000). The development of recombinant DNA technology allowed the investigators a deeper understanding of transcriptional regulation of genes and facilitated overproduction of endogenous or foreign proteins

in plants, besides unraveling the biochemical and molecular processes. A large body of literature on genetic engineering of rice is now available.

This chapter tries to cover effects of salinity on rice plant's growth and development, types of molecular mapping approaches, methodology involved in these approaches, and the achievements made through these approaches in salinity tolerance in rice to date. It also provides an overview of recent developments on the production of various transgenic lines in rice that are highly promising for abiotic stress tolerance. Thus, it will be a valuable resource for designing future research endeavors to genetically characterize salt tolerance mechanisms and develop salt-tolerant rice cultivars. It will also facilitate molecular breeding efforts for screening rice germplasm for salinity tolerance.

2 Effect of Stress on Rice Plant Growth and Development

The effects of salinity in plants are many and interfere greatly with the yield and production of the crops. Salinity affect the plants mainly by imparting two types of stresses: osmotic stress (initial stage, caused due to increased osmotic potential of rhizosphere due to high salt concentration) and ionic stress (final stage, toxicity resulted by high ionic concentration). The destructive effects of salinity include retarded plant growth due to increased Na^+ concentration (Saqib et al. 2008), delay in flowering, and impaired fertility, with partial or complete grain loss resulting in poor panicle development in rice (Abdullah et al. 2001; Kato et al. 2008; Rao et al. 2008); reduced P^{3+}, K^+, and Ca^{2+} concentrations (Fageria et al. 2012); and inhibition of photosynthetic activity (Cha-um et al. 2006; Chaves et al. 2009; Moradi and Ismail 2007).

Rice is susceptible to salinity, specifically at the early vegetative and later reproductive stages (Shannon et al. 1998). Rice genotypes show wide variations in salinity tolerance due to additive gene effects (Sahi et al. 2006). Studies indicated that rice is more resistant at reproductive and grain filling than at germination and vegetative stages (Heenan et al. 1988) and low levels of salinity can increase the resistance of rice to higher and lethal salinity levels (Djanaguiraman et al. 2006). At present, salinity is the second type of stress and is the most predominant hindrance to rice production after drought (Gregorio 1997). The effects of salinity on the growth and yield of rice in field have been well studied including the study of genotypic variance for salt tolerance among the paddy germplasms (Akbar et al. 1986; Khatun and Flowers 1995).

In addition to osmotic stress and ionic stress, plants are subjected to oxidative stress which is caused mainly due to the inhibitory action of salinity on photosynthesis. In order to cope up with the upcoming photo-inhibitory effects, plants undergo modification in their metabolic pathways such as heat debauchery by the xanthophyll pigments and electron transfer to oxygen acceptors (not water) which can result in the formation of ROS (reactive oxygen species). The later response is however mitigated by an initiation of the upregulation of several regulatory enzymes such as superoxide dismutase, ascorbate peroxidase, catalase, and the various

peroxidases (Mohammadi et al. 2008, 2013; Apel and Hirt 2004; Logan 2005). The enzymatic antioxidant defense system of plants is inclusive of superoxide dismutase (SODs), peroxidases, catalases, and the enzymes of the ascorbate–glutathione cycle: ascorbate peroxidase (APX), monodehydro-ascorbate reductase (MDHAR), dehydro-ascorbate reductase (DHAR), and glutathione reductase (GR), while non-enzymatic antioxidants include ascorbate (AsA) and glutathione (GSH) (Sharma and Dubey 2007). The oxidative damage caused is a measure of poise between the formation of ROS and its subsequent removal by the antioxidative scavenging system (Demiral and Turkan 2005). The active role of the antioxidative system has also been observed in roots of rice cultivars differing in salt tolerance (Mandhania et al. 2006). An increase in peroxidase activity in salt-tolerant cultivars under salt stress has been recorded by various researchers (Garg et al. 2002; Mishra et al. 2013; Sarkar et al. 2013). In rice, differential opinions are being established as far as oxidative responses are being concerned. Mishra et al. (2013) reported an increase in SOD activity, APX activity, and GPX activity, however reporting a decrease in CAT activity with increased exposure to salinity levels. An anticipation of the above results was however elucidated by Al-Khatib et al. (1993) where an increased CAT activity and decreased SOD and POX activity were observed in salt-tolerant lines.

3 Cross Talk of the Signaling Pathways in Rice

In response to changing normal environmental conditions, various compounds such as signaling intermediates, phytohormones, osmolytes (amino acids, sugar alcohols, tertiary amines), and some other metabolites in plant cells and tissues are accumulated and interact with one another (Gururani et al. 2015; Saed-Moucheshi et al. 2014; Arasimowicz and Floryszak-Wieczorek 2007). Recognition of the stress stimulus by cell membrane receptors induces in the cell a signaling cascade leading to the triggering of specific defense responses. In recent years, there has been an increasing interest in clarifying the role of signaling molecules in plant adaptation and protection mechanisms against environmental stress.

Recently, our knowledge about signaling mechanisms in plants starting from stimulus sensing to final response has increased. It is obvious that there are a large number of components underlying signaling mechanisms, including a high degree of interconnectivity, many spatiotemporal levels, and a complicated tune of signal transduction pathways (Fig. 1). For example, the changes at the expression of certain genes under a definite environmental condition are not necessarily translated into metabolic and structural changes where the interactions between various aspects, including posttranscriptional and posttranslational modifications, compartmentalization, metabolite stability, and substrate availability, may lead to an unexpected response (Krasensky and Jonak 2012). Moreover, it is becoming increasingly clear that signaling networks are not linear; rather they are part of a complicated and dynamic network with substantial overlap among their branches (Knight

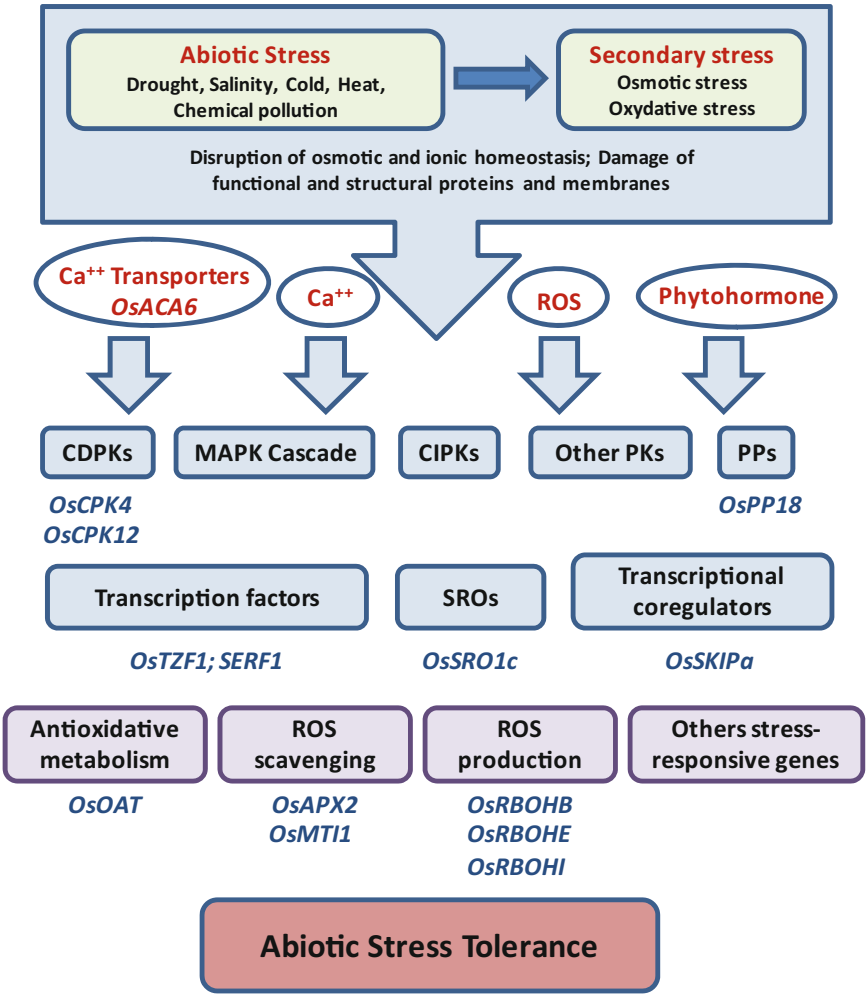

Fig. 1 Genes involved in ROS regulation and abiotic stress tolerance in rice. Plant cells perceive abiotic stress signals and transduce them through various signaling pathways including secondary signaling molecules, plant hormones, and transcriptional regulators. The regulation of gene expression by different transcription regulators results in the induction of various defense pathways, such as reactive oxygen species (ROS) scavenging and antioxidative metabolism. CDPK, calcium-dependent protein kinase; CIPK, calcineurin B-like protein-interacting protein kinase; MAPK, mitogen-activated protein kinase; PK, protein kinase; PP, protein phosphatase; SRO, similar to RCD one

2001). Accordingly, rather than one sensor, there are many sensors that perceive certain stress conditions and control all downstream signals.

During normal growth and development, ROS are produced in different cellular compartments in living cells with increased production under biotic and abiotic challenges (Moller et al. 2007; Miller et al. 2010). The traditional notion that ROS

are toxic by-products of plant metabolism has changed. Substantial experimental data are available assuring that ROS are highly controlled signaling molecules able to transfer the environmental signals, with other signaling intermediates, to the genetic machinery (Polidoros et al. 2005). Reactive oxygen species (ROS) have been shown to play an important role in plant defense mechanisms (Saed-Moucheshi et al. 2014; Kreslavski et al. 2012). It has been proposed that ROS participate as signaling molecules in the transduction of stress signals from chloroplasts to the nuclear genome and also the interactions between ROS and other signaling systems within the cell (Fig. 1) (Kreslavski et al. 2012). A rice CDPK gene, *OsCPK12*, enhances tolerance to salt stress by reducing the accumulation of ROS (Asano et al. 2012). Expression of genes encoding ROS-scavenging enzymes (*OsAPx2* and *OsAPx8*) was upregulated, whereas the NADPH oxidase gene (*OsRBOHI*) was downregulated in *OsCPK12*-overexpressing plants compared with wild-type plants. Conversely, the *oscpk12* mutant and RNAi plants were more sensitive to high salinity and accumulated more H_2O_2 than wild-type plants (Asano et al. 2012). Overexpression of another CDPK gene, *OsCPK4*, results in increased tolerance to salt and drought stresses in rice plants. Transgenic plants exhibited higher expression of numerous genes involved in lipid metabolism and protection against oxidative stress, therefore, reduced levels of membrane lipid peroxidation under stress conditions (Campo et al. 2014). The dephosphorylation mediated by protein phosphatase is an important event in the signal transduction process that regulates various cellular activities. A rice protein phosphatase 2C (PP2C) gene, *OsPP18*, was identified as a SNAC1-regulated downstream gene (You et al. 2014). The *ospp18* mutant exhibited sensitive to drought and oxidative stress with reduced activities of ROS-scavenging enzymes. The ABA-induced expression of ABA-responsive genes has not been disrupted in *ospp18* mutant, indicating *OsPP18* mediates drought stress resistance by regulating ROS homeostasis through ABA-independent pathways (You et al. 2014).

Transcriptional factors (TFs) are one of the important regulatory proteins involved in abiotic stress responses. They play essential roles downstream of stress signaling cascades, which could alter the expression of a subset of stress-responsive genes simultaneously and enhance tolerance to environmental stress in plants. OsTZF1, a CCCH-tandem zinc finger protein, was identified as a negative regulator of leaf senescence in rice under stress conditions (Jan et al. 2013). Meanwhile, OsTZF1 confers tolerance to oxidative stress in rice by enhancing the expression of redox homeostasis genes and ROS-scavenging enzymes (Jan et al. 2013).

Members of AP2/ERF (APETALA2/ethylene response factor) transcription factor family, including DREB/CBF transcription factors, are especially important as they regulate genes involved in multiple abiotic stress responses (Mizoi et al. 2012). During the initial phase of abiotic stresses, elevated ROS levels might act as a vital acclimation signal. But the key regulatory components of ROS-mediated abiotic stress response signaling are largely unknown. Rice salt- and H_2O_2-responsive ERF transcription factor, SERF1, has a critical role in regulating H_2O_2-mediated molecular signaling cascade during the initial response to salinity in rice (Schmidt et al. 2013). SERF1 regulates the expression of H_2O_2-responsive genes involved in salt

stress responses in roots. SERF1 is also a phosphorylation target of a salt-responsive MAPK (MAPK5). It activates the expression of salt-responsive MAPK cascade genes (MAPK5 and MAPKKK6) and TF genes (ZFP197 and DREB2A) (Schmidt et al. 2013).

The SRO (SIMILAR TO RCD ONE) protein family was recently identified as a group of plant-specific proteins, and they are characterized by the plant-specific domain architecture which contains a poly (ADP-ribose) polymerase catalytic (PARP) and a C-terminal RCD1-SRO-TAF4 (RST) domain (Jaspers et al. 2010). In rice, an SRO protein, OsSRO1c, was characterized as a direct target of the drought stress-related transcription factor SNAC1 (You et al. 2013). *OsSRO1c* was induced in guard cells by drought stress. Overexpression of *OsSRO1c* resulted in accumulated H_2O_2 in guard cells, which, in turn, decreased stomatal aperture and reduced water loss. Further experiments indicated that OsSRO1c has dual roles in drought and oxidative stress tolerance of rice by promoting stomatal closure and H_2O_2 accumulation through a novel pathway involving the SNAC1 and DST regulators (You et al. 2013).

4 Molecular Mapping Approaches for Improving Stress Tolerance in Rice

Molecular screening for salt tolerance in rice accounts for the recent approaches for understanding the response of rice under salt stress and thereby mines useful alleles responsible for salt tolerance (Kanawapee et al. 2011; Apel and Hirt 2004; Roychoudhury et al. 2008). The identification of saltol QTL in 2010 by Thomson et al. has widened the horizon for further work and development as far as genetic approach is being concerned (Thomson et al. 2010). Molecular mapping approaches are of three types, DNA markers, linkage mapping, and association mapping, on the basis of mapping population used.

4.1 DNA Markers

Genetic diversity analyses of several genotypes were thus employed to screen for salinity tolerance by various molecular markers: RFLP and SSLP mapping by Kanawapee et al. (2011), RAPD and SSR analysis by Ali et al. (2014), and morpho-molecular evaluation of landraces by Rikke and Johnson (1998). In order to define the complexity and the nature of the inheritance of salinity in rice, several studies were conducted which included conventional techniques such as positional cloning (Ron and Weller 2007; Bechtold et al. 1993) and "insertional mutagenesis" (Salvi and Tuberosa 2005; Rabbani et al. 2003). Several genes were identified in rice induced by high salinity levels as elaborated by Urao et al. (1999) in the course of monitoring the expression profile of rice under stress, which included genes like salT and catalase and several de novo genes.

These multiple genes governing the regulation of the response of the plants to salinity stress ensured the polygenic character of salinity as a trait. Thereby, many works were initiated to identify QTLs (quantitative trait loci) related to salt tolerance. The first breakthrough was achieved by Gregorio (1997) where a major QTL "saltol" has been mapped on chromosome I in an F8 recombinant inbred lines (RIL) population of Pokkali X IR29 cross, using amplified fragment length polymorphism (AFLP) markers. In recent years, QTL analysis in rice helped in mapping several QTLs related to several characters correlated with salinity: QTLs mapped on chromosomes 1 and 2 for shoot growth (Sabouri and Sabouri 2008), five major QTLs. qRL-7 for root length, qDWRO-9a and qDWRO-9b for dry weight root, and qBI-1a and qBI-1b for biomass (Koyama et al. 2014). Separate QTLs being identified each for sodium uptake, potassium uptake, and sodium: potassium selectivity (Lin et al. 2004), eight QTLs accounting each of three for three traits of the shoots and each of five for four traits of the roots at five chromosomal regions (Mohammadi et al. 2013) and many more. A total of 35 QTLs were identified by Mohammadi et al. (2008) in an F2 mapping population derived from a Sadri/FL478 cross, the major QTL clusters being mapped in chromosomes 2, 4, and 6 for multiple traits under salinity stress. Identification of QTL for salinity tolerance opened a new horizon in the study of salinity and the plant response to cope up with this stress thereafter. Studies are being conducted to formulate and identify different alleles whether associated with the respective QTLs for salinity (Apel and Hirt 2004; Logan 2005).

4.2 Association Mapping

Association mapping uses natural populations for mapping purposes. In this technique, commercial crop cultivars can be employed for the assessment of QTLs. Assessment of marker–trait associations is facilitated by controlling underlying population structure in the used plant material for mapping purposes (Zhao et al. 2007). STRUCTURE software is used for identifying sub-populations in the used plant germplasm (Pritchard and Wen 2004). TASSEL software is used for the identification of QTLs in this case (Bradbury et al. 2007). In recent years, association mapping is widely used to identify QTLs in plants. Association mapping approach is relatively new arrival in plant genetics. There are some reports of association mapping for salt tolerance in rice (Khan et al. 2016; Ahmadi et al. 2011; Negrão et al. 2013; Emon et al. 2015; Kumar et al. 2015; Zheng et al. 2015; Krishnamurthy et al. 2016). Main findings of these association studies are presented in Table 1. In these studies, rice mapping populations used consisted of European Rice Core Collection (ERCC) containing 180 japonica accessions (Ahmadi et al. 2011), 96 rice germplasm accessions including Nona Bokra (Emon et al. 2015), 220 rice accessions (Kumar et al. 2015), 341 japonica rice accessions (Zheng et al. 2015), 94 rice genotypes (Krishnamurthy et al. 2016), and 24 indica rice genotypes (Khan et al. 2016). Traits for which data were recorded in these studies included Na^+/K^+ ratio, survival days of seedlings, shoot K^+/Na^+ ratio, Na^+ uptake, Ca^{++} uptake, total

Table 1 QTLs identified through linkage mapping studies

Trait	Plant material used	Marker system used	Reference
Salt tolerance rating; Na^+/K^+ ratio in roots; dry matter weight of shoots	F2 population	SSR	Yao et al. (2005)
Seed germination (%); seedling root length; seedling dry matter; seedling vigor	Doubled haploid (DH) population	RFLP	Prasad et al. (2000)
Reduction rate of dry weight; reduction rate of fresh weight; reduction rate of leaf area; reduction rate of seedling height	Introgression lines	SSR	Kim et al. (2009)
Seedling survival days	RILs population	RFLP	Hongxuan et al. (1998)
Shoot length; tiller number; shoot fresh weight	Backcross inbred lines	RFLP	Takehisa et al. (2004)
Survival days of seedlings; score of salt toxicity of leaves; shoot K^+ concentration; shoot Na^+ concentration; fresh weight of shoots; tiller number per plant; plant height at the tillering stage	BC2F8 introgression lines (IL)	SSR	Zang et al. (2008)
Days to seedlings survival; score on salt toxicity symptoms on leaves; shoot K^+ concentration; shoot Na^+ concentration at seedling stage	BC2F8 advanced backcross introgression lines (ILs)	SSR	Wang et al. (2012a)
Plant height; panicle length; tillers per hill; spikelets per panicle; grain yield	RILs population	SSR	Lang et al. (2008)
Morphological and yield-related traits	F2 population	SSR	Khan et al. (2016)
Pollen fertility; Na^+ concentration and Na/K ratio in the flag leaf	F2 population	SSR	Hossain et al. (2015)
Leaf Na^+ concentration; K^+/Na^+ ratio; K^+ concentrations; ratio of leaf Na^+ to sheath Na^+ concentrations	RILs	RFLP, SSR	Haq et al. (2010)
Salt tolerance traits	RILs	RFLP, SSLP	Bonilla et al. (2003)
Plant height; root length; shoot dry weight; shoot fresh weight	RILs	SNP	Bimpong et al. (2013)
Seedling height; dry shoot weight; dry root weight; Na/K ratios in roots	RILs, *F*2:9	SSR	Wang et al. (2012b)
Sodium (Na^+) and potassium (K^+) in roots and shoots; Na^+/K^+ ratio in roots and shoots	Advanced backcross-inbred lines (BILs)	SSR	Ahmadi and Fotokian (2011)
Sodium and potassium uptake	RILs	AFLP, RFLP, SSR	Koyama et al. (2001)
Salt tolerance traits	F2 and F3 populations	RFLP	Lin et al. (2004)

cations uptake, Ca^{++} uptake ratio, K^+ uptake ratio, Na^+/K^+ uptake, and salinity tolerance scoring. Major findings made in these studies included an observation that distribution of favorable alleles associated with salt tolerance was random in ERCC (Ahmadi et al. 2011); 40 new allelic variants were found in coding sequences of five salt-related genes (Negrão et al. 2013); STS marker, RM22418, for *SKC1*, on Chr. 8 was found associated with salinity tolerance (Emon et al. 2015); region containing *Saltol* was found associated with Na^+/K^+ ratio (Kumar et al. 2015); marker RM3412 was found associated with salinity tolerance at seedling stage due to its close linkage to *SKC* gene (Krishnamurthy et al. 2016); and the report that other QTLs, in addition to *Saltol*, might be involved in salinity tolerance (Krishnamurthy et al. 2016). These reports highlighted that in rice germplasm, there might be other genomic regions involved in salt tolerance. These genomic regions need to be characterized in the future to add a wealth of information in the present rice genetics knowledge pool. Random distribution in the rice germplasm of favorable alleles associated with salt tolerance is a worthwhile finding which should be considered while exploring and selecting crossing parents in breeding programs.

4.3 Linkage Mapping

In linkage mapping, bi-parental segregating populations are used. These populations include backcross populations, doubled haploid (DH) lines, F2 populations, introgression lines (ILs), near isogenic lines (NILs), and recombinant inbred lines (RILs). JoinMap (Van Ooijen and Voorrips 2001), MapMaker (Lander et al. 1987), or QTL IciMapping (Meng et al. 2015) softwares are used for the construction of genetic linkage maps. WinQTLCartographer (Basten et al. 2001), QTL IciMapping (Meng et al. 2015), and QTLNetwork (Yang et al. 2005) programs are used for the identification of QTLs.

Linkage mapping has been very successful in the identification of QTLs linked to salinity tolerance in rice. A number of significant QTLs associated with salinity tolerance in rice were identified through linkage mapping approach (Table 2). In these studies, the mapping populations used were F2 population, F3 population, F2:4 population, near-isogenic lines, recombinant inbred lines, doubled haploid population, backcross-inbred lines, BC3F5 lines, BC2F8 advanced backcross introgression lines, and reciprocal introgression lines.

Morphological parameters are supposed to be indicators of salt tolerance. There were various reports in which QTLs related to morphological traits under salt stress were identified (Khan et al. 2016; Hongxuan et al. 1998; Yao et al. 2005; Bimpong et al. 2013; Prasad et al. 2000; Takehisa et al. 2004; Lee et al. 2007; Lang et al. 2008; Zang et al. 2008; Kim et al. 2009; Wang et al. 2012a, b). In these mapping studies, the plant material was phenotyped at the seedling, tillering, or the maturity stage. Data for different morphological traits were recorded in these studies. These traits included seed germination (%), seedling survival days, seedling vigor, seedling root length, shoot length, fresh shoot weight, dry shoot weight, dry root weight, reduction rate of dry weight, reduction rate of fresh weight, reduction rate of leaf area,

Table 2 QTLs identified through association mapping studies

Trait	Plant material used	Marker system used	Reference
Stress-responsive genes	220 rice accessions	SNPs	Kumar et al. (2015)
Salinity tolerance	96 germplasm accessions	SSR	Emon et al. (2015)
Na^+/K^+ ratio equilibrium; signaling cascade; stress protection	392 rice accessions	SNPs	Negrão et al. (2013)
Salinity tolerance	180 japonica accessions	SNPs, SSR	Ahmadi et al. (2011)
Seedling stage salt tolerance	94 rice genotypes	SSR	Krishnamurthy et al. (2016)
Survival days of seedlings and shoot K^+/Na^+ ratio	341 japonica rice accessions	SSR	Zheng et al. (2015)

reduction rate of seedling height, tiller number, salt tolerance rating, score of salt toxicity of leaves, plant height, and grain yield-related traits. A number of significant QTLs were identified in these studies. These identified QTLs included a QTL for seedling survival days (Hongxuan et al. 1998); a QTL for root length flanked by restriction fragment length polymorphism (RFLP) markers RG162-RG653 (Prasad et al. 2000); QTLs with heritability values up to 53.3% (Yao et al. 2005); two significant QTLs, *qST1* and *qST3*, for salt tolerance at seedling stage with 35.5–36.9% phenotypic variance explained values, respectively (Lee et al. 2007); the same QTLs conferring salt tolerance at both seedling and tillering stages (Zang et al. 2008); SSR marker RM223 associated with salt tolerance in rice (Lang et al. 2008); and a major QTL for straw yield, *qSY-3* (Khan et al. 2016). These studies also suggested that it is possible to combine favorable alleles associated with salt tolerance in a single cultivar through marker-assisted selection (MAS) of main effect QTLs (M-QTLs) (Wang et al. 2012a). Similarly, pleiotropic effects were found for some QTLs which were found associated with both drought and salt tolerance (Wang et al. 2012b).

There are also a number of reports of QTLs identified for different physio-biochemical traits through linkage mapping (Khan et al. 2015; Koyama et al. 2001; Bonilla et al. 2003; Lin et al. 2004; Haq et al. 2010; Thomson et al. 2010; Ahmadi et al. 2011; Hossain et al. 2015; Cheng et al. 2012; Ghomi et al. 2013). Traits which were studied in these reports were shoot Na^+ concentration; shoot K^+ concentration; leaf Na^+ concentration; leaf K^+ concentration; Na^+ uptake; K^+ uptake; Na^+ absorption; K^+ adsorption; Na^+/K^+ absorption ratio; K^+/Na^+ ratio; ratio of leaf Na^+ to sheath Na^+ concentrations; sodium (Na^+) and potassium (K^+) in roots; Na^+ concentration and Na/K ratio in the flag leaf; and sodium (Na^+), potassium (K^+), and calcium (Ca^{++}) accumulation traits. Major discoveries in these studies included a major QTL (*QKr1.2*) identified for K^+ content in the root on chromosome 1 explaining 30% of the total variation (Ahmadi and Fotokian 2011); pollen fertility, Na^+ concentration, and Na/K ratio in the flag leaf were found as the most important

attributes for salt tolerance at the reproductive stage in rice (Hossain et al. 2015); QTLs for sodium and potassium uptake were identified on different linkage groups (chromosomes) (Koyama et al. 2001) suggesting that different pathways are involved in Na^+ and K^+ uptake; and a major locus controlling Na^+ uptake (*QTLsur-7*) was identified on chromosome 7, with *R*2 value of 72.57% (Khan et al. 2015).

5 Genetic Engineering as a Prominent Method for Improving Stress Tolerance in Rice

Despite many concerted efforts all over the world, conventional breeding approaches are resulting in slow progress in developing abiotic stress-tolerant rice genotypes. One of the approaches to rectify this is to introduce genes of interest that confer tolerance to abiotic stress via genetic engineering methods. Besides, with the availability of high-quality rice genome sequence (IRGSP 2005), there has been rapid accumulation of functional genomic resources including many known cloned and characterized genes particularly for abiotic stress tolerance, genes/full-length cDNA from global expression profiles, and resequences of the rice genotypes. The identified genes are successfully transferred into rice to produce transgenics with promising traits. Transgenics are now being evaluated under field conditions in different countries. Therefore, stress-inducible genes have been utilized to improve stress tolerance through gene transfer. Although gene transformation in *japonica* rice is performed routinely in several laboratories, transformation in *indica* rice is comparatively difficult. Therefore, relatively large number of transgenic plants must be developed in *indica* species in order to select desirable transformants as well as to study the expression of introduced genes. Since the last two decades, a large number of genes were isolated and cloned which are involved in signal transduction, transcription regulation, ion transporters, and metabolic pathways.

5.1 Transcription Factors

Plant stress response is regulated by multiple signaling pathways that activate gene transcription and its downstream machinery. Transcriptome analyses using microarray technology, together with conventional approaches, have revealed that dozens of transcription factors (TFs) are involved in plant response to abiotic stress. Transcriptome data in plants suggest that there are several pathways that independently respond to environmental stresses (in both ABA dependent and independent manner), suggesting that stress tolerance or susceptibility is controlled at the transcriptional level by an extremely intricate gene regulatory network (Umezawa et al. 2006; Liao et al. 2008). Genes that have been utilized for transformation of rice till date are listed in Table 3.

Table 3 Genetic engineering of functional genes used so far for abiotic stress in rice

Nature of gene	Gene	Function	Source	Response	Reference
Transcription factor	*DREB1A*	DRE-binding protein	*Arabidopsis thaliana*	Transformants showed enhanced expression of various stress-induced genes and showed tolerance to freezing and drought stresses	Piao et al. (2001)
	cbf1	CRT/DRE binding factor	*Arabidopsis thaliana*	Transformants showed regulation of several *cor* genes at the same time and showed freezing tolerance	Zhu et al. (1998)
	cbf3	CRT/DRE binding factor	*Arabidopsis thaliana*	Transformants showed regulation of several *cor* genes at the same time and showed freezing tolerance	Prado et al. (2000)
	abi3	Abscisic acid-induced protein	*Arabidopsis thaliana*	Marked increase in expression of low temperature-induced freezing tolerance	Katiyar-Agarwal et al. (2003)
	alfin1	Member of Zn finger family protein	*Medicago sativa*	Transformants overexpressing alfin1 showed salinity tolerance	Malik et al. (1999)
Osmolyte biosynthetic genes	*OsTPP1 and OsTPP2*	Trehalose biosynthesis	Rice	Tolerance toward chilling and other abiotic stress	Roxas et al. (1997)
	bet B	Betaine aldehyde dehydrogenase	*N. tabacum*	Transformant plants showed better growth under osmotic stress conditions	Blaszczyk et al. (1999)
	p5cs	Δ1-Pyrroline 5-carboxylate synthase	*N. tabacum* *O. sativa*	Enhanced biomass under salt stress. Enhanced tolerance to salt and water stress	Villalobos et al. (2004)
	mlt D	Mannitol-1 phosphate dehydrogenase	*N. tabacum* *A. thaliana*	Enhanced tolerance to methyl viologen-induced oxidative stress. Enhanced salt stress	Garg et al. (2002) and Bryan (1990)
	*cod*A	Choline oxidase A	*O. sativa* *A. thaliana* *B. juncea*	Transformants tolerant to salt and cold. Enhanced tolerance to salt and low temperature stress. Enhanced capacity to germinate under salt stress	McKersie et al. (1996), Gupta et al. (1996) and Van Camp et al. (1996)

(continued)

Table 3 (continued)

Nature of gene	Gene	Function	Source	Response	Reference
Detoxification genes	*sod*	Superoxide dismutase	*N. tabacum* *M. sativa*	Transformants showed increased regrowth after freezing stress. Transformants showed higher photosynthetic activity during stress	Xiang et al. (2008) and Jin et al. (2010)
	Fe-sod	Fe-superoxide dismutase	*N. tabacum*	Transformants showed enhanced protection against superoxide radicals	Ding et al. (2009)
	Sat1	Serine acetyl transferase	*N. tabacum*	Transformants were resistant to oxidative stress	Liao et al. (2008)
	apx3	Ascorbate peroxidase	*N. tabacum*	Transformants showed increased protection against oxidative stress	Agarwal and Jha (2010)
	gst/gpx	Glutathione-S-transferase and glutathione peroxidase	*N. tabacum*	Transformants showed better seedling growth under chilling and salt stress	Umezawa et al. (2006)

5.1.1 ABA-Dependent Regulons

The phytohormone ABA is the central regulator of abiotic stress particularly drought resistance in plants and coordinates a complex gene regulatory network enabling plants to cope with decreased water availability (Kim et al. 2010; Saibo et al. 2009). ABA-dependent signaling systems have been illustrated as pathways that mediate stress adaptation by induction of at least two separate regulons (a group of genes controlled by a certain TF): (1) the AREB/ABF (ABA-responsive element-binding protein/ABA binding factor) regulon and (2) the MYC/MYB regulon (Uno et al. 2000). The AREB or ABFs are bZIP (basic leucine zipper) TFs that bind to the ABRE motif and activate ABA-dependent gene expressions. Overexpressing *OsbZIP23*, a member of AREB/ABF subfamily, significantly improved drought and high salinity resistance in transgenic rice at the reproductive stage (Yadav et al. 2007). Enhanced tolerance to drought and heat was observed in *35SOsAREB1* transgenic *Arabidopsis* plants (Ding et al. 2009).

5.1.2 ABA-Independent Regulons

ABA-independent well-known regulons are (1) NAC (NAM, ATAF, and CUC) and ZF-HD (zinc-finger homeodomain) regulon and (2) CBF/DREB regulon. However, in addition, several studies have identified the existence of both ABA-dependent and ABA-independent pathways of stress response that function through AP2/EREBP (ERF) family members (Gong et al. 2004).

5.1.3 The NAC (NAM, ATAF, and CUC)

The NAC family of plant-specific TFs is one of the largest in the plant genome, with 106 and 149 members in *Arabidopsis* and rice, respectively (Xiong et al. 2005; Hu et al. 2008). NAC family TFs contain a highly conserved N-terminal DNA-binding domain and a diversified C-terminal domain (Medina et al. 1999). A rice NAC gene, *ONAC045*, was induced by drought, high salt, low temperature, and ABA treatment in leaves and roots (Uga et al. 2013). The *SNAC1* overexpressing in rice seedlings showed significantly higher survival rate than wild type under drought treatment and significantly enhanced salinity tolerance as well (Nakashima et al. 2007). Hence, NAC TFs play an indispensable role in physiological adaptation for successful plant development under abiotic stress conditions.

5.1.4 CBF/DREB Regulon

The dehydration-responsive element binding proteins (DREBs) are important APETALA-type (AP2/ERF) TFs that induce a set of abiotic stress-related genes, thus imparting stress tolerance to plants. These play an important role in the ABA-independent pathways that activate stress-responsive genes. *OsDREB1A* and *OsDREB1B* were induced early (within 40 min) after cold exposure but not on ABA treatment. *OsDREB1A* was induced within 5 h of salinity stress, whereas *OsDREB1C* showed constitutive expression (Ito et al. 2006). A detailed study of all five rice *OsDREB2s* showed that *OsDREB2A* expressed to the highest levels under the control condition, and its expression was increased to some extent by high temperature, drought, and high salinity, but not by low temperature treatments.

Expression of *OsDREB2B* was markedly increased after 20 min of high and 24 h of low temperature stress. While the transcript levels of *OsDREB2C*, *OsDREB2E*, and *OsABI4* were low under the control condition, they were transiently induced by the abiotic stresses (Wang et al. 2008). Likewise, the constitutively overexpressing *CBF3/DREB1A* and *ABF3* transgenic rice showed better drought and salinity tolerance without any growth inhibition or phenotypic anomalies (Behnam et al. 2006). Overexpression of *OsDREB1F* greatly enhanced the tolerance of plants to high salinity, drought, and low temperature both in rice and *Arabidopsis*, thus playing a significant role in plant stress signal transduction (Chen et al. 2008).

5.2 Osmolyte Biosynthetic Genes

Osmolytes are synthesized in response to osmotic stress and do not interfere with normal cellular biochemical reactions. There are several examples of accumulation of osmolytes contributing to the relatively high water content necessary for growth and cellular metabolism (Dai et al. 2007). Osmolytes include proline, sugars (fructans and trehalose), polyols (mannitol and D-ononitol), quaternary ammonium compounds (glycine betaine), and tertiary sulfonium compounds. *OsTPP*1 and *OsTPP*2 are two major *trehalose*-6-*phosphate phosphatase* genes overexpressed in vegetative tissues of rice that transiently induced tolerance in transgenic rice in response to chilling and other abiotic stresses (Shima et al. 2007). Accumulation of trehalose in transgenic *indica* rice using bifunctional fusion enzyme of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase of *E. coli* has resulted in osmoregulation, removal of free radicals, and stabilization of the hydrated structure of proteins to maintain membrane integrity and protein stability under various stress conditions (Bryan 1990). Trehalose helps in maintaining individual cell structure and functions during severe environmental stress conditions. It affects sugar metabolism and imparts osmoprotection. Accumulation of proline in dehydrated plants is caused both by activation of biosynthetic pathway enzymes and by inhibition of its degradation. It has been demonstrated that overproduction of proline results in increased tolerance to salinity in transgenic rice (Wang et al. 2004).

5.3 Detoxification Genes

During stress, electrons that have a high energy state are transferred to molecular oxygen ($^1O_2$) to form reactive oxygen species (ROS) (Apel and Hirt 2004). ROS, such as singlet oxygen ($^1O_2$), superoxide ions (O_2), and peroxides, the most widely distributed being hydrogen peroxide (H_2O_2), are toxic molecules (Shikanai et al. 1998). ROS target high molecular mass molecules, such as membrane lipids or mitochondrial DNA. The toxic effects of ROS in plants are counteracted by inducing enzymatic as well as non-enzymatic antioxidative system such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), ascorbic acid (AsA), tocopherol, glutathione, phenolic compounds, etc.

The *E. coli catalase* gene (*kat E* gene) when overexpressed under CaMV 35S promoter in *japonica* rice conferred tolerance to 250 mM NaCl and enhanced the catalase activity to 1.5 to 2.5 times more than non-transgenic plants (Yadav et al. 2007).

6 Conclusions and Future Prospects

Due to the polygenic nature of the stress, it has been very meticulous to comment in particular about the exact mechanism by which mitigation of the same is achieved. However, a number of metabolic pathways, enzyme complexes, regulatory genes, and QTLs have been enumerated till date to throw some light on the various particular responses at various stages of this abiotic stress. With the help of molecular mapping approaches, a number of major and minor QTLs associated with salinity tolerance in rice have been identified in recent years, and there are further accelerated research efforts underway in this direction. The identified QTLs are valuable resources for marker-assisted selection (MAS) to develop elite salt-tolerant rice cultivars. Great task is needed to be done in this regard so that marker-assisted breeding (MAB) approach can be implemented successfully in routine breeding programs. In the future, efforts should be directed to develop climate-smart rice cultivars which can perform stably under diverse environmental conditions. Genome sequence of rice, both indica and japonica subspecies, is available now. In the next phase of annotation of the rice genome, molecular mapping results can be of help in combination with the comparative genomics approach.

Although there are many reports of transgenic rice plants with enhanced abiotic stress tolerance during field trials, further research is required to reveal the regulatory mechanisms of stress response and tolerance under field conditions. The discovery of new genes that elevate stress tolerance without yield loss under abiotic stress is very much needed. Other approaches to new gene investigation are to study stress tolerance mechanisms of stress-adapted extremophiles such as desert plants, halophilic plants.

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Conflict of Interest The authors declare that they have no conflict of interest.

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Genetically Modified Rice Stacked with Antioxidants for Nutrient Enhancement and Stress Tolerance

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Abstract

The world's population is projected to increase up to nine billion by the end of 2050, and to meet the dietary needs of this growing population, production of important agricultural crops is inevitable. Conventional breeding approaches are not only time-consuming but laborious too. Genetically modified (GM) crops can help in this regard by complementing the crop varieties developed by traditional approaches. These crops have an advantage of tackling malnutrition because of their greater potential to enhance nutritional quality and yield and an augmented resistance toward different abiotic and biotic constraints. A number of genes have been identified that can respond under biotic and abiotic stress conditions. Therefore, genetic engineering of rice for enhancing nutrient uptake and creating resistance against biotic and abiotic stresses are a novel approach for improving the agricultural crop production. Adaptation of plants to different biotic and

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abiotic stresses is contingent on creation of cascades of molecular network involving the recognition of stress, transduction of signals and expression of stress specific genes and secondary metabolites such as antioxidants. Thus, engineering the stress-responsive genes which can preserve the function may be potential target for stress tolerance and nutrient enhancement in rice.

Keywords

Genetically modified rice · Antioxidants · Stress tolerance · Nutrient deficiency

1 Introduction

Rice (*Oryza sativa*) ranks among major staple crops in the world, and it serves as a significant part of livelihood and diet of almost 3.5 billion people as the world's population is expected to cross 10 billion by the end of 2050 (United Nations 2017). Major portion of this population increase will be experienced in the developing countries where rice is mostly used as food source such as South Asia and Africa, so enhanced rice production is inevitable.

Conventional approaches for crop improvement were based upon identifying the plants showing favourable phenotypes and crossing them to get desirable results. By following these techniques, a substantial increase in the rice yield over the last half century has been observed by adopting the semi-dwarf rice varieties possessing greater resistance for lodging. Nevertheless, these techniques were not cost-effective in terms of resources, and in some rice-growing areas, they have deteriorated environmental quality through different agronomic practices such as over-use of fertilizers, pesticides and other agricultural inputs (Wing et al. 2018). Nearly 10 years ago, concept of Green Super Rice (GSR) was introduced to tackle the rice yield losses with a concurrent decrease in the cost and various ecological footprints linked with enhanced productivity (Zhang 2007). Key traits of GSR varieties were low requirements of fertilizers, pesticides and other agricultural inputs with more nutritious grains, their potential to grow on marginal lands and a decline in the greenhouse gas emissions. In addition, rice yield worldwide is affected by different biotic (insects/pests) and abiotic stresses (drought, salinity, cold, heavy metals, radioactive rays, ozone, etc.). As a result of different abiotic stresses in rice, transcript levels of different stress-receptive genes are changed. Numerous transcription factors (TFs) are actively involved in the abiotic stress response. Since, they induce several gene expressions under exposure to any abiotic stress so, they comprise of major portion of transcriptional regulatory networks termed as regulons (Singh and Laxmi 2015). Overexpression of numerous TFs such as AREBs, DREBs and NACs has led to development of abiotic stress resistance in various transgenic plants (Nakashima et al. 2009).

Over 30 years ago, transgenic plants were developed for the first time that possessed noble traits like insect and herbicides resistances (Murai et al. 1983). To achieve this objective, better understanding, managing and utilizing of genetic variation present in the domestic rice and its wild species are a prerequisite. Rice

is an ideal candidate for testing against new breeding approaches for several reasons such as rice genome is the smallest among various domestic cereals at ~400 mb and, hence, is easily amenable for genomic studies. Moreover, rice was the first plant that was completely sequenced (Matsumoto 2005), and presence of its high-quality genome allows functional, population and evolutionary genomic studies. Genes selected for adaptation and domestication can be systematically exploited for yield improvement under stressed environments.

Under exposure to various stresses, thus limiting crop yields, there is an enhanced production of different ROS which causes oxidative stress in plants leading to cell membrane abnormalities. To tackle the adverse situations, plants produce different antioxidants which may be enzymatic or non-enzymatic, and a balance begins to establish between the antioxidant production and ROS. Genetic engineering aims to impart stress tolerance to agricultural crops which is related to enhanced antioxidant production to help the plant survive under different stresses by decreasing the toxic levels of these ROS. Different studies are available in this regard, which state that an increase in the production of antioxidants assists the plant in tackling oxidative stress through declining the harmful levels of ROS in its cells and reinstating redox homeostasis (Table 1).

Many researchers have reported regarding an increased production of antioxidants in transgenic rice. Byeon and his co-workers (2014) worked on generating melatonin-rich rice plants. Melatonin is basically a bioactive molecule having enormous health-supporting properties which also include important antioxidant abilities. They, firstly, overexpressed three different tryptophan decarboxylase isogenes in rice genome independently which showed varied amount of melatonin levels in all three transgenic rice lines. Besides increasing the levels of melatonin contents in rice, they also increased the level of melatonin intermediates in rice such as tryptamine, N-acetylserotonin, serotonin, 5-hydroxytryptophan, etc. Similarly, Zhao et al. (2009) stated an increment in the actions of numerous antioxidant enzymes, e.g. superoxide dismutase, glutathione reductase, ascorbate peroxidase, peroxidase, catalase, etc., after the introduction of transcription factor YAP1 from *Saccharomyces cerevisiae* into *Arabidopsis thaliana* under exposure to salt stress. The transgenic plant exhibited lower levels of H_2O_2 which indicated lower levels of reactive oxygen species generation under exposure to salt stress. Similarly, other workers have done research in this regard. For example, Zhao and Zhang (2006) reported that overexpression of *Suaeda salsa* glutathione S-transferase (GST) gene in transgenic rice could impart salinity tolerance to rice. Similarly, Lee and Back (2017a, b) showed that transgenic rice plants harbouring N-acetyltransferase (SNAT1) (OsSNAT1) exhibited an improved production of melatonin and, resultantly, an increased tolerance toward cadmium stress in comparison with their wild types. In addition, transgenic overexpressors of SNAT1 showed better grain yield due to enhanced panicle number per plant.

Table 1 Transgenic rice stacked with different antioxidants for stress tolerance

Gene	Native species	Target species	Stress tolerance	References
Ascorbate peroxidase	*Brassica campestris*	*Arabidopsis thaliana*	Heat	Chiang et al. (2015)
	Hordeum vulgare	*Arabidopsis thaliana*	Zinc, cadmium	Shi et al. (2010)
Superoxide dismutase (SOD)	*Pisum sativum*	*Oryza sativa*	Drought	Wang et al. (2005)
	Oryza sativa	*Nicotiana tabacum*	Salinity, water	Badawi et al. (2004)
Catalase/SOD	*Brassica juncea*	*Arabidopsis thaliana*	Heat	Minglin et al. (2005)
	Oryza sativa	*Oryza sativa*	Salt/ drought	Ouyang et al. (2010)
Glutathione peroxidase	*Triticum aestivum*	*Arabidopsis thaliana*	Salinity, H_2O_2	Qin et al. (2012)
Monodehydroascorbate reductase	*Acanthus ebracteatus*	*Oryza sativa*	Salinity	Sultana et al. (2012)
	Malpighia glabra	*Nicotiana tabacum*	Salinity	Eltelib et al. (2012)
Dehydroascorbate reductase	*Oryza sativa*	*Oryza sativa*	Salinity	Kim et al. (2015)
Glutathione reductase	*Brassica campestris*	*Nicotiana tabacum*	Cold	Lee and Jo (2004)
Ascorbate peroxidase	*Solanum tuberosum*	*Solanum tuberosum*	Heat	Tang et al. (2006)
Catalase/superoxide dismutase	*Gossypium hirsutum*	*Gossypium hirsutum*	Salinity	Luo et al. (2013)

2 Genetic Engineering for Nutrient Enhancement

2.1 Engineering for N Enhancement

Nitrogen (N) plays a significant role in plant growth and development. Though N application enhances crop yield, excessive N application causes ozone layer depletion and affects global N cycle and soil leaching of nitrate (Hakeem et al. 2012). In addition, only 30–40% of the applied nitrogen is being used by the plant, and the leftover is released as gas to the environment, and as a result, N contamination degrades environmental quality (Raun and Johnson 1999). Various crops have been studied regarding variation in their genetic potential for N uptake and grain yield per unit of applied N (Presterl et al. 2003; Namai et al. 2009). Various scientists reported about genetic variation among rice genotypes regarding nutrient use efficiency (Inthapanya et al. 2000; Zhang et al. 2008). Therefore, it is imperative to develop new rice varieties that are highly efficient regarding N use (Hirel et al. 2007).

Nitrogen (N) is absorbed by the plants mainly in two forms which are ammonia (NH_3) and nitrate (NO^{3-}) via various transporter systems present in roots, and then different enzymes assimilate the absorbed nitrogen such as nitrate reductase (NiR), glutamate synthetase (GOGAT) and glutamine synthetase (GS). Glutamine synthetase (GS) is responsible for the conversion of ammonia to glutamine and has a key role in ammonia assimilation. Besides, generation of NH_4^+ also occurs as a result of different metabolic reactions such as nitrite/nitrate reduction, photorespiration and amino acid conversion (Lea and Ireland 1999). GS occurs in two forms which are cytosolic GS (GS1) and chloroplastic GS (GS2). GS1 is responsible for grain filling and normal growth, whereas GS2 has a critical role regarding the reprocessing of assimilated ammonia that is derived from different metabolic processes such as photosynthesis and photorespiration. The earlier studies indicated the critical role played by GS in nitrogen remobilization for grain filling in rice, maize and wheat (Tabuchi et al. 2007). Glutamine (Gln) is the main form of reduced N in phloem sap and is produced by GS1 during remobilization of nitrogen in cereals, i.e. rice. Many researchers have described the important role played by NADH-GOGAT1 (reduced form of nicotinamide adenine dinucleotide) in the development of sink organs in rice for reutilizing Gln. So, Gln synthesis has a key role in N cycling in rice.

Different scientists work in this regard to introduce gene of interest in different crops to check its role in enhancing antioxidant production and imparting stress tolerance in transgenic plants. For example, Yuan et al. (2015) have described that genetic modification of rice with microRNA (miR528) results in enhanced activities of catalase (CAT) enzyme in the transgenic plant and, thereby, an increase in the rice resistance to salinity as well as nitrogen (N) starvation. Tolerance to N deficiency was indicated in terms of total N accumulation, chlorophyll synthesis and overall increase in the plant biomass, activity of nitrate reductase, etc. Hence, their results elaborated the role played by miR528 in enhancing the salt tolerance and resistance to N starvation.

Similarly, in another experiment, Ge et al. (2019) introduced different transcription factors in *Arabidopsis* and rice varieties to check their effect on transgenic plants under N-limited conditions. They stated that one of the several transcription factors, SiMYB3, showed better root development under N-deficient conditions over wild types (WT). This increase was attributed to the regulation of auxin production triggered by SiMYB3. In addition, the different genes, hence differentially expressed, also participated in stress-related antioxidant production in N-deficient conditions.

2.2 Engineering for P Enhancement

Low phosphorus (P) availability is one of the major constraints to global crop production. In most soils, P is easily bound by either organic matter or other chemicals, and resultant unavailability to plant occurs which can only be made available to plants after its mineralization (Akhtar et al. 2008). P deficiency is also a limiting factor for rice growth. Plants have developed several mechanisms to cope

with P deficiency such as alteration of root morphology, cellular metabolic modifications, activation of different transporters involved in Pi transport and enhanced acquisition of P from soils by releasing low molecular weight organic acids, ribonucleases, phosphatases, phosphodiesterase, etc. (Chiou and Lin 2011). Therefore, it is of prime importance to develop such varieties that can grow under P-deficient conditions with enhanced growth and yield.

Gao et al. (2017) characterized rice purple acid phosphatase encoding gene (OsPAP26) which is a member of purple acid phosphatases (PAPs) and is specifically induced under P-deficient conditions. Overexpression of OsPAP26 resulted in a remobilization of phosphorus (P). In addition, an improvement in the ATP hydrolysis into inorganic Pi was observed in the transgenic overexpressors and application in comparison with the wild types. Their findings indicated that OsPAP26 can efficiently use rhizosphere organic P and be significantly used for crop breeding to enhance P acquisition efficiency.

Phosphate starvation response 1 (PHR1) subfamily genes play a significant role in controlling phosphate (Pi) homeostasis and Pi starvation signalling. PHR1 has three different orthologs in rice which are (Os) PHR1, (Os) PHR2 and (Os) PHR3 and have been reported to play significant part in Pi starvation signalling. Another close ortholog of PHR1 is PHR4 whose function was unknown in rice. Ruan et al. (2017) investigated the functional role of PHR4 in rice. They found that PHR4 interacts with other members of PHR1 family to control Pi starvation-mediated genes. With the establishment of knowledge regarding the positive role of PHR4 in inducing Pi starvation signalling, overexpression of PHR4 containing genotypes exhibited higher shoot accumulation of Pi and enhanced expression of Pi starvation-mediated genes under Pi-sufficient conditions.

2.3 Engineering for K Enhancement

Potassium (K) has a significant role in enormous physiological functions in plants such as enzyme activation, ion homeostasis and osmoregulation (Clarkson and Hanson 1980). Optimum plant growth is only possible under ample K^+ supply, especially K^+ transport across the endomembrane or cell membrane is related to regulation of photosynthesis. As an essential macronutrient for higher plants, it is not only important for plant growth and development, but also crucial for crop yield and quality. Due to fixation of K in soil minerals and its adsorption with soil particles, mobility of K in soil is reduced, and as a result, proportion of total K available for plant uptake is also limited in agricultural lands worldwide (Rengel and Damon 2008). Its deficiency negatively affects root and shoot growth. Its deficiency also has a depressing effect on primary root growth. In rice, K deficiency leads to a decline in the root growth, and the root to shoot ratio was also disturbed through reduction in the soluble sugar contents in the roots (Cai et al. 2012). Different genes are being identified in rice that express themselves under K starvation which involve almost 27 OsHAK genes (Yang et al. 2009). Among those, OsHAK1, OsHAK5 and OsHAK16 were significantly improved under K starvation in comparison with

other genes (Yang et al. 2014). Moreover, a shaker K channel OsAKT1 has exhibited a critical role in K acquisition by roots in rice (Li et al. 2014).

Chen et al. (2015) reported that overexpression of a WUSCHEL-related homeobox (WOX) gene (WOX11) from promoter OsHAK16 encoding a low K caused an increase in the K transporter and ultimately led to an extensive root system, adventitious roots and increase in the tiller numbers in rice. WOX11 has been reported to regulate root proliferation by controlling the auxin and cytokinin signalling. Overexpression of OsHAK16:WOX11 under moderately K-deficient soils enhanced total K uptake by 72% and grain yield by 24–32%. Their results suggested that root growth and development in WOX11 overexpressors could be used as a significant tool to enhance K acquisition efficiency under K-deficient conditions and can increase cereal growth.

Ahmad et al. (2016) assessed the impact of K^+ inward rectifying channel AKT1 on K^+ nutrition and its role in conferring osmotic and drought tolerance in rice. They characterized overexpression of OsAKT1 in rice. In their results, AKT1 expression generally correlated with K^+ uptake and tissue K^+ levels. Besides, a reduction in the plant sensitivity to drought/osmotic stress in transgenic rice lines harbouring OsAKT1 was experienced. Their results depicted that overexpression of OsAKT1 caused an increase in the tissue K^+ levels, particularly in roots, and improved osmotic and drought tolerance in rice.

2.4 Engineering for Fe and Zn Enhancement

2.4.1 Iron

One of the most prevalent micronutrient deficiencies is of iron (Fe) worldwide, and the major cause is its reduced absorption from different cereals. Fe deficiency results in anaemia which affects almost two billion people worldwide. In this regard, pregnant women and children are, particularly, more susceptible. Anaemia, in turn, can cause a reduction in physical and cognitive development as well as a decline in immunity. It also enhances the risk of perinatal and maternal mortality (Stevens et al. 2013). Recommended average daily iron intake varies from 8 to 18 mg/day based upon body weight, age and gender, whereas 30 mg/day is recommended for pregnant women (WHO 2016).

Fe performs numerous important functions such as chlorophyll biosynthesis, photosynthetic electron transport and respiration. Its deficiency causes a reduction in the chlorophyll contents of young leaves (Marschner 1995). It is also involved in the normal functioning of mitochondria and chloroplast. Fe is transported to mitochondria via different mitochondrial Fe transporters (Bashir et al. 2011). Surplus Fe is diverted to vacuole for storage (Zhang et al. 2012a, b).

2.4.2 Iron Biofortification in Food Crops

To date, genetic engineering and conventional breeding strategies possess the potential to deliver nutritious Fe-rich food to malnourished populations. For this purpose, germplasm containing the trait of interest must be available to the breeders.

Despite all of this, traditional breeding techniques sometimes, alone are not able to enrich the micronutrient contents in plants up-to the nutritionally recommended levels. This failure may be attributed to lack of natural genetic variation or negative correlations between micronutrient concentrations and grain yields (Amiri et al. 2015). Among cereals such as wheat and rice, endosperm has been engineered with iron transport and storage and, hence, proved to be a promising approach. For this, plants have been modified using ferritin expressed under the control of endosperm-specific promoters, and as a result, an enormous increase in the iron contents of polished rice grains and wheat flour has been observed (Oliva et al. 2014). Ferritin, however, alone was not successful in achieving the targeted iron levels in neither rice nor wheat. Rather, parallel transformation of rice with genes relating to phytosiderophore synthesis has also shown its potential regarding the enhancement of iron contents up to their targeted levels (Johnson et al. 2011; Lee et al. 2012). Moreover, iron biofortification in rice has also shown considerable success via introducing combination of different genes such as ferritin and nicotianamine synthase (NAS) and other important genes involved in encoding iron transporters (Masuda et al. 2012; Aung et al. 2013). The following are the list of different examples of biofortification of Fe in rice using different biotechnological strategies: (1) storage, (2) efficient uptake and translocation, (3) regulation of deficiency responses, (4) intercellular and intracellular storage and transport and (5) combination strategies (Table 2).

Nowadays, new technologies have emerged that have proved to be very promising in Fe biofortification programme such as reverse breeding, mutagenesis, RNA-directed DNA methylation, genome editing, etc. (Schaart et al. 2015). Moreover, different genomics methods and molecular breeding programmes are now being combined to achieve specific traits such as association genetics, marker-assisted selection genotyping and high-throughput phenotyping (Ricroch et al. 2016).

2.4.3 Zinc

Zinc (Zn) plays a significant role in all life forms (Broadley et al. 2007), and it can bind with more than 900 proteins in the human body (Oliver and Gregory 2015). According to the Food and Agriculture Organization and the World Health Organization (FAO and WHO 2004), reference nutrient intake (RNI) for Zn in adult males and females is 14 and 10 mg capita 1, respectively, whereas, the requirements for youngsters are even more. Zn deficiency in children increases the severity and incidence of diarrhoea and enhances the risks of stunted growth (Mayo-Wilson et al. 2014). Body of an adult human comprises nearly 2 g of Zn out of which 30% is present in bone mass and 60% is present in skeletal muscles (Saltzman et al. 1990).

Fe and Zn are both essential elements for plant growth, and they also fall among the list of essential micronutrients that are required by plants for their normal growth and development. In different low-income scenarios, where the utilization of animal food source is less, plants serve as the primary source for dietary Fe and Zn. There are different factors which influence the quantity of Fe and Zn contained in different

Table 2 Transgenic rice for iron biofortification

Target	Gene (s)	Fe increase as compared to control	Rice cultivar	References
Storage	GmFerritin	Up to 3 folds	Rice/Japonica cv. Kitaake	Goto et al. (1999)
	GmFerritin	Up to 3.7 folds	Rice/Indica IR68144	Vasconcelos et al. (2003)
	OsFerritin	Up to 2.09 folds	Rice/Indica Pusa Sugandhi	Paul et al. (2012)
	GmFerritin H1	Up to 3.4 folds	Rice/Indica cv. IR 64	Oliva et al. (2014)
Uptake and translocation	HvNAS1	Up to 2.3 folds	Rice/Japonica cv. Tsukinohikari	Masuda et al. (2009)
	OsNAS1	2.2 folds	Rice/Japonica cv. Nipponbare	Johnson et al. (2011)
	OsNAS2	Up to 4.2 folds	Rice/Japonica cv. Nipponbare	Johnson et al. (2011)
	OsNAS3	2.2 folds	Rice/Japonica cv. Nipponbare	Johnson et al. (2011)
	OsYSL2	Up to 4.4 folds	Rice/Japonica cv. Tsukinohikari	Ishimaru et al. (2010)
	HvIDS3 genome fragment	1.4 folds	Rice/Japonica cv. Tsukinohikari	Masuda et al. (2008)
Deficiency response	OsIRO2	>2 folds	Rice/Japonica cv. Tsukinohikari	Ogo et al. (2011)
Intercellular and intracellular storage and transport	OsVIT1or OsVIT2 functional disruption (mutants)	~1.5 folds	Rice/Japonica cv. Zhonghua 11 and cv. Dongjin genetic background, respectively	Zhang et al. (2012a, b)
	OsVIT2 T-DNA insertion line	>1.5 folds	Rice	Bashir et al. (2013)
Combination strategies	*PvFERRITIN*, *AtNAS1* and *AfPHYTASE*	Up to 6.3 folds	Rice/Japonica cv. Taipei 309	Wirth et al. (2009)

plant organs such as plant type and its variety, soil type and growing environment along with its management.

Zn is also involved in different metabolic processes (Ishimaru et al. 2011). Nucleic acid, protein, lipid and carbohydrate metabolism depend on Zn uptake to a great extent (Rhodes and Klug 1993). Zn uptake should be strictly regulated to certify that its desired amounts are always present (Ishimaru et al. 2011). Its

deficiency causes the build-up of inactive RNases and starch, indicating that RNA degradation is controlled by Zn availability in the cell (Suzuki et al. 2012).

2.4.4 Factors Affecting Fe and Zn Bioavailability

One of the primary causes of Fe and Zn deficiencies is inability of diet to supply enough of these elements. Sometimes, possibility is there that sufficient quantities of Zn and Fe are consumed, but due to some antinutrients present in the diet or some other physiological reasons, absorption of those micronutrients is declined. Moreover, their bioavailability may also be affected by other factors such as phytate component of the diet makes insoluble complexes with Fe and Zn and cause resultant inhibition of their captivation by intestine of human. Humans are monogastric animals, and, hence, they lack phytate-degrading enzymes such as phytase in their stomach (Hurrell and Egli 2010). Diets having inadequate bioavailable Zn are defined as having a phytate to Zn molar ratio of more than 15 (Gibson et al. 2010). Fe is present in non-haem forms in plant tissues, and its bioavailability is reduced by phytate, tannins, polyphenols and other important components (Hurrell and Egli 2010). On the contrary, ascorbate may enhance Fe bioavailability by reducing ferric to ferrous and acting as a chelate (Siegenberg et al. 1991).

2.5 Engineering for Other Micronutrient Enhancements

The human body needs nearly 22 essential elements, some of which are needed in larger quantities, whereas some elements such as zinc (Zn), copper (Cu), iodine (I), iron (Fe) and selenium (Se) are required in smaller concentrations. Higher concentrations of these elements in body are harmful for normal body functioning (Welch and Graham 2004; Broadley and White 2009). It is stated that out of total world population of six billion, nearly 30% are Zn deficient, 15% are deficient in Se, 30% are iodine deficient and 60–80% are Fe deficient (Combs 2001). There are different factors that are responsible for this deficiency, and as a result, malnutrition occurs in the population. For example, less consumption of vegetables, fruits, fish and other animal products and more utilization of staple food such as cereals, more consumption of processed food and sowing of crops in the areas having low phyto-available mineral contents, etc. lead to their deficiency in the diet. Moreover, one important factor is the less availability of these minerals in the human consumable tissues of plants.

Biofortification techniques involving different approaches such as biotechnology and plant breeding, etc. are not new to this era; rather, they were used to mediate nutrient deficiencies nearly a decade ago in food crops (Ye et al. 2000). It is a cheaper and effective alternative to conventional ways of tackling deficiencies of micronutrients, i.e. miconutrient supplementation and food fortification. However, these methods are costly, and majority of the world's population cannot afford them, particularly those having low incomes and limited resources (Haas et al. 2005). Grain biofortification to combat nutrient deficiency possesses great potential and imparts a drastic effect on human health in this regard.

3 Approaches to Cope with Stress

3.1 Conventional Approaches

Plant breeding techniques have a long history of adoption for generating salt-tolerant and high-yielding rice cultivars. It has been shown that some conventional varieties of rice such as Bura Rata, Nona Bokra, Pokkali, etc. are better at tolerating salinity than many bulging varieties. Pokkali has been extensively used in breeding programmes as a gene donor to generate salt-tolerant varieties. Better salt tolerance in Pokkali is attributed to not only its ability to preserve low Na^+/K^+ ratio in plants as well as its faster expansion rates under salinity. Many salt-tolerant rice varieties have been prepared worldwide through breeding such as CSR10, CSR13, CSR27, IR2151, Pobbeli, PSBRc84, PSBRc (48, 50, 86 and 88) and NSIC106.

However, breeding for promoting salt tolerance using wild germplasm is really challenging because of their reduced agronomic characteristics (tallness, photosensitivity, poor grain quality and low yield) despite having enhanced salt tolerance. Another issue in conventional breeding approach is the reproductive difficulty that if the gene is present in the wild equivalent of the crop, plant breeder may face trouble in its introduction to domesticated variety. So, modern approaches have been preferred to develop salinity tolerance in rice.

3.2 Genetic Engineering for Stress Tolerance

3.2.1 Engineering for Salinity Tolerance

Salinity is one of the major abiotic stresses that badly affects the crop production worldwide, particularly for arid to semi-arid areas where the annual precipitation is less than the evapotranspiration. To tackle this peril, two broad approaches were utilized in the late twentieth century. First was the reclamation of salt-affected soils, and the second was the introduction of "halophytes". The second approach was termed as "biotic approach". Scientists classified the biotic approach as cost-effective, easy and more efficient in terms of bringing salt-affected soils into use (Ashraf 1994). With the world population reaching 9.6 billion by the end of 2050 (UNFPA 2014), crop productivity must be increased by 44 million metric tons annually. This is rather challenging to expand the arable lands, whereas the climatologists predict that a greater portion of land will be subjected to abiotic stresses and erratic environmental conditions globally (Eckardt 2009; Cominelli et al. 2013). Salt stress confines rice yields especially at mature stage (Todaka et al. 2012). Yield losses in rice under the influence of salts have been estimated as 30–50% annually (Eynard et al. 2005).

Salt stress induces a variety of inhibitory effects in terms of plant growth such as a decline in the net rate of CO_2 assimilation, dry matter accumulation, cell enlargement in leaves and overall leaf growth (Amirjani 2010). Munns (2002) reported a reduction in ability of rice to uptake essential nutrients and water from the soil and an increase in the metabolic alteration under salt stress. In addition, poorly developed

rice spikelets, particularly inferior spikelets, as a result of salt stress also led to a significant decrease in grain yield (Zhang et al. 2016). Excess amount of sodium (Na^+) in plants also harms cellular organelles and cell membrane, and as a result, physiological mechanisms of plants are disturbed which ultimately leads to cell death (Siringam et al. 2011). Among the harmful physiological changes are disruption of cell membrane, oxidative stress due to inability of the plants to detoxify reactive oxygen species (ROS), reduction in the net photosynthetic rate and alterations in the antioxidant enzymes (James et al. 2011). These changes can interfere with the normal functioning of the different components of the cell, for example, DNA, lipids and proteins, as well as interrupting active cellular functions in plants (Demiral and Türkan 2005).

Salinity resistance in plants is controlled by variety of host genes and is a quantitative character (Chinnusamy et al. 2005). In recent times, many genes that confer salinity tolerance in different cereals especially rice have been recognized and categorized in this regard, such as those responsible for signal transduction, ion transportation, metabolic homeostasis and transcription regulation (Kumari et al. 2009).

However, significant achievements have been made to impart salt tolerance in rice through genetic manipulation. Mutation breeding, however, has proved to be the most important approach regarding the production of high-yielding salt-tolerant rice varieties (Flowers 2004). It possesses a great potential regarding the production of salt-tolerant varieties with high-yielding abilities (Das and Roychoudhury 2014). Many researchers have reported an increase in the production as well as salt tolerance of rice cultivars subjected to mutation breeding.

Similarly, Nath et al. (2015) isolated a gene PDH45 from pea DNA, which is an exclusive member of helicase family and is responsible for imparting salinity tolerance, and inserted it to transgenic rice to assess the effect of PDH45-assisted salinity tolerance in rice. They used CoroNa green dye to check Na^+ localization in plant roots and shoots, and their results indicated that transgenic rice containing PDH45 exhibited less root and shoot accumulation of Na^+. However, root accumulation of Na^+ was more as compared to its accumulation in shoots in transgenic rice varieties, which showed that Na^+ movement to shoots was inhibited, and it was proposed that PDH45 might be involved in the deposition of apoplastic hydrophobic barriers and, resultantly, caused a decline in the translocation of Na^+ to shoot and, thereby, conferred salinity tolerance to PDH45 expressing transgenic rice varieties. In addition, the extent of ROS production in transgenic plant was also reduced as compared to their concentration in wild types. So, oxidative stress was also mediated in the PDH45 overexpressors in comparison with the wild types. Reports are available regarding the role of PDH45 in controlling ROS production and protection of photosynthetic machinery of rice, thereby imparting salinity tolerance to the plant (Gill et al. 2013). They also elaborated that salinity-sensitive (Pusa-44) and salinity-tolerant (FL478) varieties of rice accumulated high and low Na^+, respectively.

To date, the focus of the genetic engineering approaches is the genes encoding for antioxidant production, osmolyte production, ion transport, heat shock proteins, etc.

Here are some of the examples of genes for conferring salinity tolerance and enhancing antioxidant production in rice (Table 3).

3.2.2 Engineering for Drought Tolerance

Frequent occurrence of drought and limited water availability threaten agricultural safety (Lobell and Field 2007; Mickelbart et al. 2015). The USA suffered an agricultural drought in 2012, and as a result, nearly 12% of reduction in corn production was documented in comparison with the previous year (USDA 2014). In Asia, almost 34 million hectares of lowland rainfed rice and 8 million hectares of upland rice experienced drought stress (Abbas et al. 2014; Mustafa et al. 2015).

Generally, drought stress causes yield reduction in plants by shortening their life cycle and decreasing dry weight accumulation (Blum 2005; Kamoshita et al. 2008). During vegetative growth, drought stress can cause a decline in growth and development of different organs involved in photosynthesis, especially well before the onset of flowering (Okami et al. 2015). It can also limit pollen viability, stigma receptivity and seed setting (Barnabás et al. 2008).

Drought stress affects rice growth and development at all stages; however, water stress interferes with grain formation in rice during flowering stages (Boonjung and Fukai 1996). This reduction in yield is attributed to a decrease in panicle exertion and spikelet fertility (Ji et al. 2005). The decrease in the panicle exertion is mainly due to inhibition of peduncle elongation that can cause nearly 70–75% sterility in spikelets under water-deficient conditions (O'Toole and Namuco 1983). Inhibition of reproductive organ development such as ovary and pollen at meiosis (Saini 1997) along with process inhibition, e.g. anther dehiscence, pollen shedding, pollen germination and fertilization (Ekanayake et al. 1990; Satake and Yoshida 1978) due to drought have been recorded. An ideal root system can maximize water capture, and, hence, moisture reaches at greater soil depths; therefore, plant growth under drought stress is improved (Asch et al. 2005). Genotypes with shallow-rooted crops have less yields as compared to deep-rooted genotypes under drought stress (Kato et al. 2011).

In recent times, numerous attempts have been carried out to engineer drought tolerance in plants by relocating various functional genes of interest that are involved in the active encoding of enzymes responsible for production of transporters, scavengers of reactive oxygen species (ROS), chaperones and other osmotically active compounds (Vinocur and Altman 2005). Many genes responsible for the synthesis of various compatible solutes such as amino acids, quaternary and other amines (polyamines and glycine betaine) and different alcohols (mannitol, galactinol, etc.) and sugars that get accumulated during osmotic adjustment have been introduced to date (Vinocur and Altman 2005). One of the important approaches in this regard is the discovery of a novel pathway of glycine betaine synthesis, which is a major osmoprotectant in salt-loving microorganisms (Waditee et al. 2005).

Rahman et al. (2016) isolated and characterized a transcription factor NAC67 from finger millet (*Eleusine coracana* L.) and analysed its role through *Agrobacterium*-assisted genetic transformation in a rice cultivar ASD16. Overexpression of NAC67 in transgenic rice lines exhibited enhanced tolerance of

Table 3 Transgenic rice stacked with antioxidants for salinity tolerance

Gene	Protein	Gene source	Development stage	Results	References
CAT1 and GST	Catalase and glutathione S-transferase	*Suaeda salsa*	Germination, vegetative	Enhanced salt tolerance and increased CAT, GST and SOD activities, decreased H_2O_2	Zhao and Zhang (2006)
Gly II	Glyoxalase II	*Oryza sativa*	Vegetative	Enhanced salt tolerance, increased glyoxalase activities	Singla-Pareek et al. (2008)
GS2	Chloroplastic glutamine synthetase	*Oryza sativa*	Vegetative	Increased salt tolerance and less Na^+ accumulation	Hoshida et al. (2000)
katE	Catalase	*Escherichia coli*	Vegetative	Enhanced salt tolerance and catalase activities	Moriwaki et al. (2008)
Mn-SOD	Mitochondrial manganese superoxide dismutase	*Saccharomyces cerevisiae*	Vegetative	Enhanced salt tolerance and SOD activities	Tanaka et al. (1999)
Sod1 dismutase	Cytosolic copper zinc superoxide	*Avicennia marina*	Vegetative	Higher salt tolerance	Prashanth et al. (2008)
$CamV_{35}S$	G-protein γ subunit	*Oryza sativa*	Vegetative	Increased salt tolerance and CAT, APX and GR activities	Swain et al. (2017)
OsSUV3	SUV3 protein	*Oryza sativa*	Vegetative	Enhanced CAT, APX, GR and proline and decrease in H_2O_2	Tuteja et al. (2013)
PDH 45	PDH45 protein	*Oryza sativa*	Vegetative	Enhanced SOD, GPX, GR and APX activities	Gill et al. (2013)

Table 4 Transgenic rice stacked with antioxidants for drought tolerance

Gene	Crops	Stress	Antioxidants	References
OsABA8ox3	*Oryza sativa*	Drought	Enhanced SOD and CAT activities and decreased malondialdehyde (MDA)	Cai et al. (2015)
OsCPK10	*Oryza sativa*	Drought	Enhanced CAT and decreased H_2O_2	Bundó and Coca (2015)
C_4 pepc gene	*Oryza sativa*	Drought	Enhanced ascorbate peroxidase (APX) activities	Qian et al. (2015)
AtWRKY57	*Oryza sativa*	Drought	Increased SOD, CAT and POX activities	Jiang et al. (2016)
OsCML4	*Oryza sativa*	Drought	Increased CAT, SOD and proline activities	Yin et al. (2015)
OsNAC6	*Oryza sativa*	Drought	Increased glutathione and nicotianamine synthesis	Lee et al. (2017)

rice toward drought stress under greenhouse conditions. Shoot and root biomass of transgenic rice was found to be higher, and plants showed better revival upon stress relief. Similarly, high relative water contents and less yield reductions were also observed in transgenic rice as compared to wild types.

Similarly, Fang et al. (2015) studied the effect of SNAC3—a stress-reactive NAC gene in rice under drought, temperature and oxidative stress. Overexpression of SNAC3 in rice caused improved tolerance toward these stresses. Moreover, suppression of SNAC3 reversed the results. Transgenic lines overexpressing SNAC3 exhibited a significantly lower level of malondialdehyde, H_2O_2 and electrolyte leakage, when compared with the wild types.

Lee et al. (2017) used OsNAC6, a key regulator of stress response in rice, and evaluated its role in protecting rice from the harmful impacts of drought. Their results showed that overexpression of OsNAC6 containing transgenic rice lines exhibited less reductions in grain yield than the wild types. OsNAC6 also upregulated the regulation of different target genes involved in nicotianamine biosynthesis, membrane modification, glutathione relocation and glycosylation that indicate multiple drought tolerance pathways. Xiong et al. (2018) reported the role played by ERF family transcription factor OsLG3 on regulation of rice growth under drought stress. In their experiment, overexpression of OsLG3 caused an improvement in drought tolerance of rice plant. However, this improvement was more evident in upland rice as compared to low land rice. Further studies revealed OsLG3 caused ROS scavenging in rice, and a resultant increase in drought tolerance was observed.

In addition, in different experiments, transgenic rice varieties which were genetically modified using maize C4 *pepc* gene showed better antioxidant production capacity and an improvement in the nitrogen use efficiency under exposure to drought stress (Qian et al. 2015). Here are some of the genes responsible for enhancing drought tolerance and antioxidant production in rice (Table 4).

3.2.3 Engineering for Heavy Metal Stress Tolerance

In the past few decades, there has been an increase in the industrialization, urbanization, fossil fuel burning, anthropogenic activities, and as a result, threshold levels of different heavy metals in the plant-soil system have been augmented, and all life forms have been seriously damaged (Shahid et al. 2017; Ashraf et al. 2019). Excessive and non-judicious use of hazardous chemicals (pesticides, fertilizers, municipal solid wastes, herbicides, fungicides) in agriculture for enhanced production, industrial effluents, metalliferous mines and energy and fuel production, etc. has further worsened the situation (Bardiya-Bhurat et al. 2017; Ashraf et al. 2019).

Harmful impacts of heavy metals on plants are usually the same which involve low biomass production, growth inhibition, a shift in the water and nutrient balance, chlorosis, yield reduction, etc. In addition, they are also a threat to human life because of their longer retention time in environment (Mitra 2015). Moreover, high concentration of heavy metals in the soils and plants also enters the food chain and subsequently to the human body and causes many harmful diseases (Ashrafzadeh et al. 2018). This issue may further aggravate if serious immediate measures are not taken (Murtaza et al. 2015). So, it is of greater importance to devise such methods to decrease the entrance of these trace metals in different crops through soil and their subsequent entrance in the food chains (Ashraf et al. 2019).

Mostofa et al. (2015) evaluated the role of trehalose (Tre) in enhancing rice tolerance against copper (Cu) stress. They pretreated rice seedlings with Tre and significantly alleviated the harmful effects of Cu on the photosynthesis and other growth-related parameters. This enhancement in heavy metal tolerance was attributed to its ability to uptake lower Cu levels and a decline in the Cu-induced oxidative stress via lowering of ROS accumulation and malondialdehyde in Cu-stressed plants. Besides, Tre reduced the Cu-induced increase in glutathione and proline contents, but increased redox status and ascorbate contents. In addition, Tre treatment also enhanced the activities of major antioxidants in rice exposed to Cu stress. For example, glyoxalase I and II activities were enhanced with a concurrent decline in the methylglyoxal levels. Similarly, Pan et al. (2018) isolated a 12 HsCIPK from the Tibetan Plateau annual wild barley and analysed its role in imparting tolerance against heavy metal toxicities. Their results indicated heavy metal (Cd, Pb, Cu, Hg, Cr, etc.) toxicity caused the upregulation of HsCIPK. Overexpression of HsCIPK in transgenic rice lines showed enhanced tolerance of rice to heavy metal toxicities. Their results showed that HsCIPK has a significant role in conferring heavy metal tolerance in rice.

Moreover, there are several genes that have been reported to be involved in Cd uptake and tolerance in rice, and many researchers have reported on this regard (Takahashi et al. 2012). Besides, Cd-excluder rice varieties have also been developed. A Cd-excluder can survive in higher concentrations of Cd without accumulating it (Zhan et al. 2015) (Tables 5 and 6).

Table 5 Genetic engineering of rice for heavy metal tolerance

Plants	Gene	Heavy metal	References
Oryza sativa	Phytochelatin synthase (PCS) gene *OsPCS1*	Cadmium (Cd)	Li et al. (2007)
Oryza sativa	Serotonin *N*-acetyltransferase (*SNAT1*) OsSNAT1	Cadmium (Cd)	Lee et al. (2017)
Oryza sativa	Yeast cadmium factor 1 (YCF1) gene	Cadmium (Cd)	Islam and Khalekuzzaman (2015)
Oryza sativa	OsSUV3	Cadmium (Cd) and Zinc (Zn)	Sahoo et al. (2014)
Oryza sativa	Arsenite (AsIII) antiporter gene PvACR3	Arsenic (As)	Chen et al. (2019)
Oryza sativa	Ubi::MIR528	Arsenic (As)	Liu et al. (2015)

Table 6 Transgenic rice stacked with antioxidants for heavy metal tolerance

Gene	Heavy metal stress	Results	References
MTH1745	Mercury (Hg) stress	Enhanced superoxide dismutase and peroxide, decrease in the H_2O_2 and malondialdehyde	Chen et al. (2012)
OsLEA4	Manganese (Mn)	Increased proline and decreased malondialdehyde activities	Hu et al. (2016)
SNAT gene	Cadmium (Cd)	Increased melatonin activity and decreased malondialdehyde activities	Lee et al. (2017)
C4 PEPC and PPDK	Aluminium (Al) stress	Enhanced activities of CAT and SOD, decreased activities of malondialdehyde	Zhang et al. (2018)
OsPCR1 and OsPCR3	Cadmium (Cd)	Increased activities of CAT, POD and SOD and decrease in H_2O_2 production	Wang et al. (2019)
OsSultr1;1	Arsenic (As)	Increased glutathione contents and decreased malondialdehyde, H_2O_2 contents	Kumar et al. (2019)

3.2.4 Engineering for Pest and Diseases Tolerance

3.2.4.1 Insect-Resistant Rice

Development of insect resistance in crops has modernized agriculture, has decreased the reliance of insecticide usage with a safeguard to the environment and human health and has proved to be a major tool in the integrated pest management (IPM) programmes (Brookes and Barfoot 2013). Insect resistance in rice developed nearly four decades ago. Nowadays, genetically altered rice harbouring gene from *Bacillus thuringiensis* (Bt rice plants) are under examination worldwide (Wang et al. 2014a, b). There are various reports that transgenic rice expressing Bt gene exhibit the tendency to minimize crop losses incurred by the attack of lepidopteran pest in Asia (High et al. 2004). Effectiveness of Bt rice was also assessed in filed conditions

Table 7 Genetic improvement of rice for insect resistance

S. no.	Gene(s)	Targets	References
1	cry1Ab or cry1Ac	YSB[a], SSB[b]	Shu et al. (2000)
2	cry1Aa or cry1Ab	SSB	Breitler et al. (2004)
3	cry1Ab and cry1Ac	YSB	Ramesh et al. (2004)
4	cry1Ab	SSB	Cotsaftis et al. (2002)
5	cry1Ab	YSB and RLF[c]	Bashir et al. (2005)
6	cry, Xa21, and RC7	YSB, bacterial blight, sheath blight	Datta et al. (2003)
7	gna and cry1Ac	Homopteran, coleopteran andlepidopteran insects	Nagadhara et al. (2003)
8	Itr1	Rice weevil	Alfonso-Rubí et al. (2003)
9	cry1Ac and cry2A	YSB and RLF	Rahman et al. (2007)
10	Bt and CpT1	Insect resistance	Rong et al. (2007)
11	Bt, protease inhibitors, enzymes and plant lectins	Insect resistance	Deka and Barthakur (2010)
12	cry2Aa	Insect resistance	Wang et al. (2012)
13	cry1Ab	Insect resistance	Wang et al. (2014a, b)

[a]YSB = yellow stem borer
[b]SSB = stripe stem borer
[c]RLF = rice leaf folder

against attacks of lepidopteran, and they also exhibited resistance to their attacks (Deka and Barthakur 2010; Wang et al. 2014a, b).

Similarly, transgenic rice hybrids were examined in a field trial in China, and they showed high tolerance to insect attack such as rice leaf folder (RLF; *Cnaphalocrocis medinalis*) and YSB (Chen et al. 2011). Insect-resistant rice varieties have also been developed in Pakistan (Rahman et al. 2007), and they have shown tremendous potential against YSB and RLF. Similarly, transgenic rice have also been developed in China, and their efficiency in the field as well as lab conditions has been evaluated (Li et al. 2016) (Table 7).

3.2.5 Genetic Engineering of Rice for Resistance to Diseases

3.2.5.1 Fungal Diseases

Rice plant is vulnerable to different pathogens such as viruses, bacteria and fungi, so genetic enhancement of rice is of prime importance (Sattari et al. 2014). Many genes were isolated and reintroduced in rice to confer resistance against different species of fungi in the past. For example, Pi-ta gene was found to have a considerable role in developing resistance against rice blast (Delteil et al. 2010). Similarly, PR-3 rice chitinase gene was found to be imparting tolerance in rice against sheath blight (Datta et al. 2003). Overexpression of Rir1b in transgenic rice has proved to be significantly enhancing resistance in rice to blast (Li et al. 2009).

Moreover, many proteins in rice have also been discovered that possess the tendency to develop resistance in rice against several fungal species, such as lipid

Table 8 Transgenic rice stacked with antioxidants for disease resistance

Gene	Fungus	Disease	Results	References
Rice chitinase gene (RCH10)	*Rhizoctonia solani*	Sheath blight disease	Enhanced SOD activities	Mao et al. (2012)
alfalfa â-1,3-glucanase gene (AGLU1)	*Rhizoctonia solani*	Sheath blight disease	Enhanced SOD activities	Mao et al. (2012)
Rice xylanase inhibitor protein (RIXI)	*Magnaporthe oryzae*	Rice blast	Increased SOD, POD and CAT activities and decreased H_2O_2 concentration	Hou et al. (2015)
Cajanus cajan hybrid-proline-rich protein encoding gene (CcHyPRP)	*Magnaporthe grisea*	Rice blast	Enhanced activities of SOD and CAT and reduced malondialdehyde levels	Mellacheruvu et al. (2016)
Osa-miR308b gene	*Magnaporthe oryzae*	Rice blast	Increased SOD activities	Li et al. (2019)
Simultaneous use of rice heterotrimeric gamma subunit of rice G-protein (RGG1) and beta subunit of G-protein (RGB1)	*Rhizoctonia solani*	Sheath blight disease	Enhanced CAT, APX and GR activities	Swain et al. (2019)

transfer proteins (Guiderdoni et al. 2002), puroindoline proteins (Krishnamurthy et al. 2001), mycotoxin detoxifying compounds (Higa et al. 2003), protease inhibitor protein genes (Qu et al. 2003), antifungal proteins from *Aspergillus flavus* (Coca et al. 2004), trichosanthins (Yuan et al. 2002), selenium-binding protein homolog (Sawada et al. 2004), defensins (Kanzaki et al. 2002), phytoalexins (Lee et al. 2004) and genes taking part in cell death (Matsumura et al. 2003) (Table 8).

3.2.5.2 Bacterial Diseases

Bacterial blight is caused by pathogen *Xanthomonas oryzae* pv. *oryzae* in domestic rice (*Oryza sativa*). Transgenic rice having the resistance against bacterial blight was developed by transforming an endogenous gene Xa21 which is assumed to be the best candidate for inducing resistance against bacterial blight in rice. It is also thought that Xa21 also helps in developing multiple stress tolerance (Datta et al. 2003).

Wang et al. (2013) reported molecular cloning of avirulent gene, designated as avrXa23, from *Xanthomonas oryzae* pv. *oryzae* (Xoo), which invades the host rice plant through injecting a myriad of effector proteins and causes the bacterial blight in rice. They authorized that avrXa23 effector is responsible for triggering broad-spectrum resistance in rice isogenic line CBB3 against bacterial blight, as CBB3 has shown to be resistant against almost all the strains of Xoo. So, their result depicted that avrXa3 is responsible for conferring resistance in CBB3 against

bacterial blight as the presence of avrXa3 was documented in all the 38 strains of Xoo tested.

Vo et al. (2018) screened a WRKY gene, OsWRKY67, whose expression is enhanced under pathogen attack, and found that it considerably enhanced resistance in transgenic rice lines against two pathogens, i.e. *Xanthomonas oryzae* and *Magnaporthe oryzae*. Overexpression of OsWRKY67 gene in rice caused enhanced tolerance to diseases.

3.2.5.3 Viral Diseases

Reductions in rice yield as a result of viral attack have been a serious global issue especially rice dwarf virus (RDV) and rice stripe virus (RSV) that caused enormous yield losses especially in the 1960s. Insecticides can be a suitable option to kill the vector insects, but high cost of insecticides and their environmental impacts limit their use. So, the best alternative is to develop genetically engineered rice varieties to cope with the problem of viral infections. Rice plants showing resistance against RSV (Xiong et al. 2009) and RDV (Sasaya et al. 2014) have been developed.

Rice tungro disease (RTD) is one of the serious viral diseases limiting rice production especially in tropical Asia and is a result of an interaction between *Rice tungro bacilliform virus* (RTBV) and *Rice tungro spherical virus* (RTSV). RSTV resistance prevailing in conventional rice cultivars has led to a decline in the field RTD incidence. Naturally existing RTSV resistance is a recessive trait which is regulated by eukaryotic translation initiation factor 4 G (eIF4G). Macovei et al. (2018) generated a mutation in the eIF4G using the CRISPR/Cas9 system in order to develop new source of resistance to RTD in RTSV susceptible variety IR64, which is extensively grown in tropical Asia. With a gene mutation of 36–86.6%, some of the mutated eIF4G alleles conferred resistance to the transgenic line.

Rice black-streaked dwarf virus (RBSDV) belongs to family *Reoviridae* and genus *Fijivirus* and is the cause of severe yield reductions in Asia. RNA silencing has been quite successful in engineering rice plants against viral attacks. For example, Wang et al. (2016a, b) developed transgenic rice variety possessing a hairpin RNA (hpRNA) construct focusing on four RBSDV genes (S1, S2, S6 and S10) responsible for encoding RNA-dependent RNA polymerase, putative core protein, RNA silencing suppressor and outer capsid proteins, respectively. All the transgenic lines exhibited strong resistance to RBSDV infection.

3.2.5.4 Engineering for UV Radiation Tolerance

Sunlight generates a variety of radiation spectrum, of which part approaching the earth surface comprises infrared, ultraviolet (UV-A and UV-B) and visible light, among which, UV-B, having the wavelength of 280–315 nm, exerts numerous structural effects on living organisms (Singh et al. 2017) such as alteration in the structure of proteins, DNA, lipids and other important biological molecules, increased production of reactive oxygen species (ROS) and inhibition of several important physiological processes such as photosynthesis (Babele et al. 2015; Chudobova et al. 2015). In addition, they are involved in the direct damage to DNA via encouraging the formation of dimers in the cellular DNA such as

pyrimidine (6-4) pyrimidinone photoproducts and cyclobutane pyrimidine dimers (Santos et al. 2013).

In contrast to this, few researchers reported a non-significant change in the physiology of plant under exposure to UV-B up to 30% elevation above the ambient level of UV-B radiation (Chimphango et al. 2003). So, it was concluded that harmful effects of UV-B are also associated with other factors such as dosage, intensity, cultivars, plant species and environmental conditions, etc. (Hui et al. 2014). Rice plants cultivated between 40°N and 40°S latitudes are particularly affected by UV-B radiation (Mohammed and Tarpley 2011), and response varies from species to species and among growth stages.

3.2.5.5 Engineering for Ozone Tolerance

Despite the international policy efforts to combat the global change, combustion of fossil fuel is on the rise globally and will continue to do so for the coming decades, particularly from developing countries (IPCC 2014). This situation poses serious threats to agricultural production worldwide, which nowadays faces a challenge to feed the population of nine billion by the end of the twenty-first century, of which almost 8 billion will be living in the less developed countries of the world (United Nations 2014). Fossil fuel burning as part of anthropogenic activities is a major source of air pollution which affects both plant growth and human health. Troposphere ozone is the most widely recognized air pollutant that affects crop growth (Van Dingenen et al. 2009). In addition, ozone also blocks photosynthesis through stomatal closure and loss in activity of Rubisco (a photosynthetic enzyme; Wilkinson et al. 2012). Moreover, ozone exposures on changing the crop quality have also been reported, i.e. an increase in the protein contents of cereal and reduction in the feed value of important forage crops (Wang and Frei 2011). Ozone exerts its harmful effects on rice yields via decline in spikelet numbers, grain size and spikelet fertility. In addition, it also alters the rice grain and straw quality. So, there is a need to develop ozone-resistant rice varieties.

Ashrafuzzaman et al. (2017) used ethylenediurea (EDU) as a screening tool to check the response of rice upon ozone exposure. They applied EDU in controlled conditions on three contrasting rice varieties. One was tolerant to ozone stress, and the other two were sensitive to ozone exposure. The results indicated ozone badly affected all the plant growth and physiological parameters such as root/shoot lengths, SPAD value, chlorophyll contents, stomatal conductance, etc. However, these harmful effects were more evident in ozone-sensitive varieties. EDU application showed no effect on plant growth in the absence of ozone stress. However, EDU responses were more visible in sensitive cultivars than in tolerant ones. They also stated that EDU has no constitutive effect on rice growth and it only alleviated the harmful effects of ozone up to a certain limit with more pronouncing effect on sensitive genotypes.

Frei et al. (2010) performed a study to check whether the negative effects of ozone on rice growth can be alleviated through molecular breeding. They used three rice genotypes, and four different levels of ozone were used in a fumigation chamber, and growth was checked from transplantation till maturity. Two of the

rice genotypes were ozone sensitive, whereas the third cultivar was an ozone-tolerant genetically engineered rice cultivar harbouring QTLOzT9 gene responsible for ozone tolerance. Their results indicated that ozone negatively affected rice growth. Ozone-tolerant genotype containing QTLOzT9 was less responsive toward ozone stress than the sensitive genotypes. So, it was proved that QTLOzT9 was involved in mitigating the harmful effects of ozone in rice.

3.2.5.6 Genetic Engineering for Cold Stress Tolerance

Cold stress is also another important abiotic stress in rice and retards plant growth at all life (from vegetative to reproductive) stages. Cold stress may decline growth and development of rice, i.e. growth reduction in seedlings, tiller numbers and plant height during vegetative growth stages (Tian et al. 2012). It causes incomplete panicle exertion, delayed heading, pollen sterility, a decline in the rate of seed setting, poor grain development and, ultimately, a reduction in yield (Pan et al. 2015). Different plants have grown numerous methods for tolerating low temperature stress. Based upon signal transduction routes, cold-tolerant responsive genes can be separated into three types, viz. transcription factors, functional genes and protein kinase genes. For example, ICE-CBF-COR plays a significant role in conferring cold stress resistance to plants (Wang et al. 2017). Several cold-responsive genes (COR) are expressed under exposure to cold stress. Numerous scientists have characterized C-repeat binding factors (CBF) resembled genes in rice too (Miura and Furumoto 2013). Similarly, Ito et al. (2006) reported that OsDREB1 expressing transgenic rice exhibited an improved resistance against cold, salt and drought stress.

Zinc finger proteins have a significant contribution in imparting resistance against environmental stresses in several plants (Zhang et al. 2015). Among them, C2H2 zinc finger protein plays an important role regarding abiotic stresses resistance to plants. So, Jin et al. (2018) used a C2H2 zinc finger protein transcription factor OsCTZFP8 in rice which encodes for C2H2 zinc finger protein. Expression of OsCTZFP8 was significantly induced by cold stress. Overexpression of OsCTZFP8 caused an improved resistance to cold stress concurrent with higher pollen fertility and seed setting rates than their wild types. Similarly, yield per plant in OsCTZFP8 overexpressors was also more as compared to the non-transgenic lines under low temperature stress.

Similarly, DREB subfamily also plays an important role under salt stress tolerance. Moon et al. (2019) characterized an unidentified member of DREB family "OsDREB1G" which is induced under exposure to low temperature stress. This gene is predominantly functional in leaf blade, sheath, root and node. Transgenic overexpressors of OsDREB1G showed an improvement in the plant resistance to cold stress. Cold receptor genes were dominantly in transgenic rice harbouring OsDREB1G under low temperature stress than the wild types. Hence, OsDREB1G is a characteristic DREB1/CBF, exclusively for cold stress tolerance. So, OsDREB1G has proved to be an important tool imparting cold tolerance in rice. Here are some of the examples of transgenic rice with improved antioxidant production under cold stress (Table 9).

Table 9 Transgenic rice stacked with antioxidants for cold stress tolerance

Gene	Source	Results	References
Serotonin *N*-acetyltransferase (SNA)	Human genome	Enhanced melatonin levels in plants	Kang et al. (2010)
OsAPXa	*Oryza sativa*	Less H_2O_2 levels and malondialdehyde contents. Enhanced ascorbate peroxidase (APX) activities	Sato et al. (2011)
$OsDREB_2B$	*Oryza sativa*	Increased glutathione peroxidase, catalase, glutathione reductase, glutathione activities	Wang et al. (2013)
OsDREB6	*Oryza sativa*	Enhanced proline and catalase activities	Ke et al. (2014)
2-Hydroxymelatonin (2-OHMel) gene	*Oryza sativa*	Enhanced proline activities	Lee and Back (2016)

4 Concluding Remarks and Future Perspectives

Genetically modified crops are helping the world to fulfil the increasing food demand without compromising the quality in a very efficient manner. Several stress tolerance genes have been used to construct GM crops stacked with antioxidant to combat stress tolerance. However, further studied are needed to carefully identify the vital components of the plant stress response. Moreover, development of nutrient-rich crops like finger millet, cassava, etc. without any toxic product will be remarkable. Taking in mind the social and regulatory issues related to GM crops, existing trait modulating technologies such as clustered regularly interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases genome editing approaches should be used to meet the concerns of consumers. Surely, this will helpful in developing the superior quality crops and allow the commercialization even in countries where transgenic crops are poorly accepted.

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Breeding and QTL Mapping for γ-Oryzanol and Nutrition Content in Rice

Anirban Roy and Somnath Bhattacharyya

Abstracts

With the increasing demand for healthy dietary plan for human food consumption, cheaper strategy for developing countries is to feed human population with high nutrition and providing quality demand of developed country where consumption is less with higher amount of nutrition. Most of the breeding objectives of rice surrounds its genetic improvement for yield and making it sustainable for changing environmental dynamics. Breeding rice variety for nutrition is in the juvenile phase though crop improvement programs towards this direction have been started since the inception of modern breeding tools. Breeding for Zn, Fe, and other mineral dense rice to eliminate nutrition-deficient hunger through the exploitation of available genetic variability from the close or wide gene pool and utilizing modern genomic tool can shape nutrient-rich rice breeding. At present breeding for oryzanol is a target for industry as this wonder compound makes rice grain a good source of antioxidant and improves the quality of rice bran oil which makes this rice by-product closer to the industry as well as health-conscious people. The quantitative nature of these target traits need to exploit the genomic region through map-oriented breeding introgression of QTLs from donner source to popular cultivars with minimum genetic drag involving selection using a large set of codominant markers would be an easier approach. Genome-wide selection using new-generation markers linking character would be finer strategy to exploit evolutionary association of target characters with any phenotype and breeding towards gene identification, and genome reconstruction can help towards rice medicine.

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1 Introduction

In the context of gearing global population around 9–10 billion, it is mandatory to increase food availability to feed this burgeoning human population (Godfray et al. 2010; FAO 2017). Among cereal-based food sources, rice is a staple food for more than nearly half of the world's population. Still now more than 3.2 billion people are undernourished with good food sources, and to eliminate the deficiency in nutrition status among people, breeding for rice variety should be oriented towards with good amount of product with better nutrition through selective breeding approach or through genetic improvement (Gearing 2015). Consumption of rice is a habit of people since long years ago, but nutrient nourishment through different forms through rice consumption is way forward. It has been estimated that rice supply 715 kcal/capita/day, 20% dietary energy in developing countries which exceeds up to 50% dietary energy consumption through only rice in countries like Bangladesh, Cambodia, etc. (Kennedy et al. 2004), though several countries of the world fully depend on rice for food availability because of soul source (Pellett and Ghosh 2004) and very less diversity (Zhu et al. 2007). Nowadays countries from less developed part of the world are trying to nourish people through staple food enrichment because this is the cheaper strategy (Mahender et al. 2016). Breeding rice variety for nutrient-rich rice must be undertaken to encounter hidden hunger in nutrition in Asian countries (Swamy et al. 2016). Breeding strategies should be high yielding nutrient rich with bioactive compounds having beneficial role to human health to get rid of hunger and malnutrition among the rice-consuming people of several parts of the world to prepare a supply chain of nourishment. Micronutrients which are very much important from the view of essentiality for fine functioning of health status and iron, zinc, iodine and β-carotene have been identified as major vulnerable stage as influencing more malnutrition (Welch and Graham 2004). Rice grain is a good source of B vitamins like riboflavin, niacin and thiamine and deficient in β-carotene and vitamin A. Most nutrition factor-oriented problems, occurring in the regions, depend on rice-based meal solely to avoid protein–energy malnutrition and iron and other nutrients and essential component deficiencies which leads to death of numerous children, affected by malnutrition, which constitutes to nearly five million annual child death below the age of 5 (Shrimpton et al. 2001). But variability for carotene content in rice is nil and as a result needs to engineer by recombinant DNA technology (Tan et al. 2004). Components of quality-rice mainly count protein contents, cooking time, glycaemic index and high Fe-Zn-rich as Zn is a cofactor for more than 300 enzymes including acting as a co-ordinating ion and amino acid-enriched and antioxidant-enriched rice having tocotrienol, tocopherol, γ-oryzanol (Tripathy et al. 2017), etc. Tocotrienol and tocopherol are vitamin E homologues, present in rice bran, help as a remedy of cardiovascular

disease (by improving plasma cholesterol level towards good cholesterol more), inflammatory disease, cancer and macular disease acting as antioxidant (Qureshi et al. 1996). γ-Oryzanol, a natural product, is a scavenging agent of diphenylpicrylhydrazyl (DPPH), hydroxyl and superoxide radicals and protects cells against lipid peroxidation in during oxidative stress in cell (Juliano et al. 2005). As a bioactive compound, γ-oryzanol has the most promising value in the nutritional aspects that can enrich rice nutritional status and gaining a different status in marketing as rice bran oil enriched with γ-oryzanol by surpassing other cooking oil in India as well as in western countries. Most of the bioactive components of quality rice resides in the pericarp-outer layer, but milling permits loss of bioactive components and this compelled to consume whole-grain products. A List of quality parameters of rice also includes the colour of grain which varies from black to red including different shades (Goufo and Trindade 2014). Other important nutritional traits representing a group of antioxidant property includes phenolic acids, flavonoids, anthocyanins and proanthocyanidins, phytic acid, etc. In general red and black kernel rice contains flavonoids imparting the red colour, proanthocyanidin and anthocyanin, respectively. Gallic acid, a common antioxidant, is highly present in red rice. Fe, Zn, Ca, Cu and Mg present in higher quantity in black, red and purple-coloured indigenous cultivars like folk rice and can be donor sources for nutrient enrichment in modern rice breeding. As a whole fraction of protein that is supplied from rice-based food varies near to 40% and 3.8%, cereal-based lysine in rice enriches the protein content (Shobha Rani et al. 2006). People from America and Africa are now more interested in antioxidant-rich rice with better nutritional value and exploiting much of their resources to find better enhancement. Rice, enriched with aspartic and glutamic acid but-in general deficient in lysine and tryptophan (FAO 1993) which need to be rectified in seed storage protein though they are not possible in a conventional way, need to dissect SNP variation and can be exploited for high amino acid-rich rice. Best conventional crop improvement is based on selection of dense nutritive value genotype and using that genotype as a donor for popular cultivar. On the other way utilizing quality and nutrition-rich gene pool from wild species like *Oryza nivara* and *Oryza rufipogon* can be helpful to boost genetic diversity for exploiting variability in breeding (Ma et al. 2016; Swamy et al. 2014). Recently, 'Chattisgarh Zn Rice-1' and 'BRRI Dhan 62' with Zn content more than 20 mg/kg in polished rice have been released (Singha et al. 2017). QTL detection for amino acid enrichment also can be exploited where QTL for lysine and other amino acid has been developed (Wang et al. 2008). Breeding for these quality traits mapping of these genes is essential as for quick response marker-assisted breeding is more time efficient. Assisting conventional breeding with the integration of markers like SSRs (Gande et al. 2014), SNPs (Mammadov et al. 2012) and STSs (Chandel et al. 2011) causes selection at an early stage. Through QTL approach identifying the contributing loci or group of loci needs to be confirmed and fine-tuning of that position so that multiple traits contributed by a QTL can be improved or enhance the interaction component, and finding QTL for multiple nutrient elements from major genomic regions and identification of candidate genes would be the best strategy through fine mapping QTL region using close flanking marker

for foreground selection. QTLs for phytate concentration having antioxidant role also helps in early seedling growth (James et al. 2013). Involving the genomics approach for a complex traits, where environmental influence causes a complex inheritance patterns is essential nowadays. In this present context genomics-oriented technology will lead to nutrient-rich grain for a better future of nutrition status (Perez-de-Castro et al. 2012). Utilizing modern genomic tools including microarray, transcriptome, genome-wide association and genome-assisted breeding will lead to develop a superior rice variety with high nutrition status (Varshney et al. 2014). Though the downstream process for commercializing a transgenic output is a time-bound series of events, still imparting the biotechnological approach needs to be careful because of stringency in downstream process in varietal improvement and establishment and can be utilized efficiently when the question regarding better nutrition for under nourished nation comes. In a view for enhancing global nutrition status through HarvestPlus, food biofortification programme including rice crop utilizing scientific community from different institutions all over the world has been geared up (Andersson et al. 2017). But the use of rice as a staple food in Asia is decreasing in an urban scenarios that's why it is important to increase the nutritional aspects of rice for continuing its consumption in Asia (Kim et al. 2013a, b). So, it is most important to breed rice that can benefit as medicinal input. Most of the modern cultivars, except recently developed few cultivars, has lower amount of Zn and Fe, but traditional varieties are hidden source for these traits implies that in recent rice variety development programme, breeding for nutrition was not a major objective though recently gained a momentum (Zuo and Zhang 2011). So, now exploiting rice breeding in this direction is very much needed. Exploiting the QTL approach for breeding nutrition-dense rice variety is ideal as this technology has been used in other cereal crops as well as fine-tuning for candidate region. The advantage of using QTL mapping in this simpler genome in comparison to other major crops are for finding target genomic region and within gene variation that can be exploited for selection easily. Way-out for breeding this kind of traits having complexity regarding inheritance in rice, that information will lead to obtaining genetic inheritance and selection strategy through the conventional phenotypic selection and on the other hand availing markers for genes which contribute to improved nutritional quality rice, and identification of donor rice varieties having hidden or unexploited potentiality for those genes towards target traits, involving indirect selection strategy of crop improvement programme through molecular markers. To fulfill this objective, answers to the following questions are being attempted:

1. What are the major traits need improvements?
2. What is the nature of inheritance?
3. Which part of the genome controls target trait?
4. What should be the selection strategy?
5. What markers are available for indirect selection for traits?
6. What are the underlying genetic mechanisms?

2 Nutritional Deficiency of Rice

Several parts of the world take nutrition only through rice due to poor accessibility of other sources, but rice is also gives insufficient amount of several nutrients resulting in serious malnutrition in these developing countries (Sautter et al. 2006). Protein content of rice is not so high like other several cereals instead of its larger consumption. Rice is also relatively deficient in several amino acid of dietary requirement to maintain nutrition-specific value of some proteins. There is need to change in protein sequence and enhancing quantity of some amino acid in storage proteins targeted for seed, though this breeding goal needs sophisticated technology like involving codon optimization and transgenic development or genome-wide SNP identification. Not only deficiency, the form of rice we consume consists of a lower amount of nutrients. It has been deduced that brown rice contains Fe 10–11 ppm and Zn 20–25 ppm where in milled rice it varies from 2 to 3 ppm and 15 to 17 ppm, respectively, i.e. 70% has been reduced after milling causing a dietary deficiency for these nutrients (Sellappan et al. 2009). Quantity of folate (B9) in rice is very little or absent totally, for its improvement, folate enrichment transgenic technology in rice with significant folate improvement for rice biofortification has been taken.

3 Rice Grain

Rice grain consists of pericarp, tegmen (seed coat), aleurone layer, endosperm and embryo with different amount contributing towards a complete grain (Delcour and Hoseney 2010). After hulling the rice grain composition reduced to 6–7% bran, around 90% endosperm and unchanged 2–3% embryo if not broken (Chen et al. 1998) called brown rice. Among different rice important ones are parboiled rice, polished rice, basmati rice, puffed rice, organic rice, wild rice and two by-products which are rice bran and rice flour (Verma and Shukla 2011). Rice bran is a by-product obtained after rice milling during whitening which is a waste product and underutilized till now. But modern technology exploited rice bran for its several beneficial compounds like nutritional component and medicinal property including a higher amount of protein, lipid, vitamins and minerals (Issara and Rawdkuen 2016). The use of rice bran nowadays is industry-based where extraction of rice bran oil having balanced fat composition is most promising including its value in pharmaceutical and cosmetic industry (Friedman 2013). Chemical composition of rice consists of 70–75% carbohydrate, 7–10% protein and 1% lipid that are the major thing nutrition status previously decided for rice grain (Lee et al. 2014).

3.1 Nutrient Contents in Rice Grain

Rice grain has essential micronutrients like Fe, Zn, Ca, etc. in varying amount. Major component of rice nutrition is carbohydrate (72%), and the rests are protein (7–10%) and lipid (1%). Rice contributes about 40% protein uptake for human (Tripathy et al.

2017). Other components of nutritive value of bran of rice consist of biomolecule like protein, vitamins, minerals, phenolics, oxidation scavenging role and vitamin E derivative compound, bioactive compound of high nutritive value (Renuka and Arumughan 2007). Rice bran has several nutraceutical values. The nutrient content of cooked rice depends on storing period after harvest, especially, the aroma of cooked rice reduce remarkably irrespective of the varieties. Even after cooking protein content is reduced by less than 7% (Lee et al. 2014). The most promising bioactive components that enrich rice nutritional value are γ-oryzanol. Efforts to increase iodine and selenium content in rice have been started simultaneously with other component also (Graham et al. 2001).

3.2 Antioxidant Component of Rice

Antioxidant property of rice is not so high except bioactive compound oryzanol and few polyphenolic contents, which are present in high amount in rice. Antioxidant amount varies between white rice and coloured rice as the maximum part that possesses antioxidant property is present in outer covering of grain (Goufo and Trindade 2014). It has been reported that phenolic acids contain more antioxidant property than anthocyanins compound in rice bran (Min et al. 2011). In case of reducing power, higher amount is present in phenolic compounds, whereas α-tocopherol poses lower amount (Laokuldilok et al. 2011). Among the components of bioactive compound γ-oryzanol, the maximum antioxidant property was shown by 24-methylenecycloartanyl trans-ferulate component (Xu et al. 2001).

3.3 Glycaemic Index of Rice

Food composed of carbohydrates changes blood sugar level and can be quantify through the measure of glycaemic index after food consumption. GI as a component of rice quality eases the digestion and absorption by the cell causing a change in blood sugar levels. Digestion and absorption in slow pace indicates lower glycaemic index having foods and permits need-based release of sucrose into the blood circulation, maintaining a balance inside the system. GI varies from 54 to 121 in all the available rice phenotyped (Manay and Shadaksharaswamy 2001). GIs of 55 or less are considered as 'low', those of 56–69 are medium, and those of 70 and above are 'high' (Brand-Miller et al. 2000). A detailed dissection of 235 rice line from different corners of the world, by international institutes like IRRI and CSIRO under the food-based programme, found that the glycaemic index changes according to rice varieties ranging from near 50 to more than 90, where majorly consumed rice grain having GI range from low to medium GI. This implies rice impart health benefit for the average consumer.

3.4 Phenolic and Flavonoids

In general the phenol property stabilizes and delocalizes free unstable electrons and provides a stability to these compounds, where these property solely depends on quantity and its orientation of OH group (Heuberger et al. 2010). Among 12 identified phenolic acid ranging around 7–8 mg/100 g in the endosperm, with higher amount in bran like average 248 mg/100 g, 20.8–78.3 mg/100 g has been represented by whole grain and near to 500 mg/100 in the husk (Irakli et al. 2012). Among several phenolic acids *p*-coumaric acid, sinapic acid, gallic acid and protocatechuic acids are common, but a major amount is represented by ferulic acid (Deng et al. 2012).

3.5 Lipid Content

Low lipid content in rice from storage context is good, but now high lipid containing rice is also popular in industry based-by-product of rice like rice bran oil production. Thus complex breeding objective and genetic mechanism necessitated understanding lipid content optimization towards two diverse goals (Qin et al. 2010). Lipid content in rice having relatively high amount of heritability (60.90–68.25%) (Qi et al. 1983). It has been established that these lipids reduce serum and LDL cholesterol and simultaneously increases HDL cholesterol levels (Qureshi et al. 1991). Lipid components from rice have been identified as food-derived functional compounds and has been reported its inheritance as quantitative trait controlled by number of genes (Wang et al. 2006). Lipid content is controlled by several QTLs, chromosome 1, 2, 5 having total three major QTLs associated in this direction (Hu et al. 2004). Protein content of rice also depends on environment as it differs in seasons with dose and time of nitrogenous fertilizer application. Recently NRRI, India, developed two high yielding rice varieties with high protein content viz., CR-2829-PLN-37 (CR Dhan 310) and CR-2829-PLN-100 (CR Dhan 311/Naveen) in the background of 'Naveen' variety (Chattopadhyay et al. 2019). Initially two QTL for rice protein content have been identified, one near to waxy gene which explains 13% variations and another QTL within R1245-RM 234 interval (Tan et al. 2001). QTL mapping for protein content in this case have helped a lot for developing high protein rich rice; two major consistent QTL identified viz qGPC-1 and qGPC-10 (Yang et al. 2019), where qGPC-10 harbor OsGluA2 controls variation in both japonica and indica rice. Recently, three QTL viz. qPC1.1, qPC1.2 and qPC7.1 have been identified which are colocalized with serine, histidine and threonine amino acid content QTL in rice (Jang et al. 2020). Through association mapping, one QTL for protein content has been identified (Zhao et al. 2011) previously; besides, three novel QTL viz. QTLs qPC3.1, qPC5.1 and qPC9.1 have been identified through association mapping (Pradhan et al. 2019).

4 Breeding for Protein Content

As compared to other cereals like wheat, barley, etc., amount of protein present in rice grain is very low. Around 25% of children suffer from protein malnutrition in the areas where diets are based on cereal like rice with fewer amounts of essential amino acids (Gearing 2015). Though in developing countries 40% protein of total intake comes from rice and attracts breeders target for improving grain protein content (Shobha Rani et al. 2006).

5 Vitamins and Minerals

Milling procedure causes a substantial loss of nutrient from outer layer of kernel. Wide range of vitamins suffers loss in the grain around 50–80% including thiamin (B1), riboflavin (B2, niacin (B3), biotin (H), pantothenic acid (B5), pyridoxine (B6), folic acid (B9) and vitamin E and in case of other macromolecules like proteins by around 10–16% and fats around 80% and fibres around 60% average loss during milling process effecting a huge nutrient drain out (Dexter 1998).

6 Breeding Strategies for Nutrition Content

Allele mining in available genetic resources can be exploited to introgress better allele with positive effect. Incorporating new-generation technologies of popular cultivars can yield a better nutrition-rich product (Peng et al. 2016). To reduce the time needed for conventional cross-breeding programme as like mini-maize, at first, nutrition donor can be crossed with short duration (<80 days) variety and then transferring to other commercial cultivars (McCaw et al. 2016). Positive effect multiple QTLs can be incorporated to engineer a promising cultivar with multiple nutrient traits that can be easily deliverable to the nutrition chain (Mahender et al. 2016). QTLs/genes pyramiding from multiple backgrounds with minimum genetic drag started at a good pace by marker-assisted technology in a reasonable time.

6.1 Oryzanol Component in Rice

Bioactive compounds are able to act as scavengers against superoxide radicals (Wang et al. 2002) those generate during different stress and used to damage several biomolecules (Liu 2007). Besides oryzanol, rice bran contains tricin, found to be oxidative stress scavenging compound (Ajitha et al. 2012; Poulev et al. 2019). γ-Oryzanol is a carboxylic acid-alcohol-based ferulate with sterols or terpenoid alcoholic property (Xu and Godber 1999). Numerous advantages of γ-oryzanol in rice cause modern rice breeding towards enriching rice nutritional factors with this bioactive compound. It has been identified that γ-oryzanol promotes growth related to reproductive hormones and also stimulates the releasing organ (Rogers et al. 1993;

Xu and Godber 1999; Akihisa et al. 2000) overall. γ-Oryzanol has the property for improving the stability of pharmaceuticals, i.e. acts as natural antioxidant and used as food preservatives (Juliano et al. 2005). γ-Oryzanol is present in rice from the USA to European countries (Kato et al. 2017). Amount of γ-oryzanol varies among species and intra-specific varieties where amount is more in japonica rice than the indica type. Total oryzanol content varies from 3400 to 4200 mg/kg (Chen and Bergman 2005; Bergman and Xu 2003), and in the varieties popular in European countries, varies from 260 to 630 mg/kg (Miller and Engel 2006), while 201–388 mg/kg is found in some cultivar available in Venezuela (Aguilar-Garcia et al. 2007). In many studies regarding rice bran oil enrichment, it has been found that vitamin E, oryzanol component, etc. constitute this rice bran, with potential effectiveness to lower serum-cholesterol level, maintaining a healthy plasma cholesterol through radical scavenging property (Tsuji et al. 2003).

6.2 Breeding and QTL Mapping for Oryzanol Content

Available genetic variation in nature for this compound in different rice cultivars and the higher content of γ-oryzanol in Asian rice including γ-tocotrienol are important for breeders' perspective for selection of genotypes with enriched phytochemicals and for identification of responsible genetic factor and QTLs which can be used in breeding programme. Phenotyping for γ-oryzanol revealed constituents of oryzanol (mg/kg of brown seed rice) in various varieties of rice where the presence of four different components (Zubair et al. 2012) of oryzanol including cycloartenyl ferulate, 24-methylene cyclo artanyl ferulate, campesteryl ferulate and sitosteryl ferulate identified through various fractionation and chromatography methods those of has been found. Studies on variability in γ-oryzanol content is limited and hence underlying genetic mechanism and even for phenotyping of a limited number of samples analysed, leads difficulty in understanding the genetic mechanism regarding the variability in *γ*-oryzanol content. Variability in the composition of steryl ferulates in brown rice is unexploited as *γ*-oryzanol's ferulate component influencing antioxidative and cholesterol lowering effect, where genetic mechanisms need to engineer for further improvement (Miller and Engel 2006). An initial preliminary linkage map was constructed from one RIL and one BIL derived from both indica and japonica rice using MAPMAKER/EXP 3.0 (Lander et al. 1987); QTL analyses were conducted based on Windows QTL Cartographer ver.2.5 for overall γ-oryzanol quantity in a RIL- and BIL-based population using indica and japonica (Wang et al. 2007), with LOD score around 3. Various QTL-based candidate regions for oryzanol content flanking by SSR markers have been identified in substitution line population flanking by XNpb13 andR2638 in chromosome 9 with increasing allelic effect from IR24 and also other candidate region flanking by XNpb37&C405, C1069&R1684, RM8105&RM3233, RM3534&RM2431 (129.6) 6 RM1370&RM5463, RM3773&RM6673, 11 RM5824&RM1355 and RM6905&RM3813 (87.6) most of the allelic affect from genotype 'Sasanishiki' and 'Habataki'. Study towards QTL analysis in the direction for enhancing quantity

of bioactive compound, total eight QTLs like qOZ1c, qOZ5a, qOZ1a, qOZ1b, qOZ5b, qOZ7, qOZ12 and qOZ9 for improving γ-oryzanol content in brown rice, in various mapping populations have been identified. In another study, QTLs for nutritional factors like oryzanol which enhance the value of grain and bran oil has been detected in the background of chromosome segment substitution line derived from that cross combinations involving Sasanishiki as one parent, while it has been pointed that two out of all the regions identified from different cross combinations involves similar loci (Kato et al. 2017). Detailed study further revealed that detected QTLs and the one involving candidate loci for the target trait represents all the key molecule that constitutes complete anabolic pathway of γ-oryzanol (Piironen et al. 2000). The region identified as QTL holds some genes for protein coding for oryzanol biosynthesis pathway which includes Δ14-sterolreductase (Locus Id: Os01g25189) within *qOZ1b* qtl region, cycloartenol synthase (Locus Id: Os05g14800) near to *qOZ5a*, sterol14-demethylase (Id: Os05g34380) near to *qOZ5b* and sterol14-demethylase (Id: Os07g37980) where a *qOZ7* is present nearby and another demethylase enzyme related to steroyl within the same region (Kato et al. 2017).

6.3 Genetic Resources for Nutrient Content

A high Zn content rice has been reported recently from IIRR (Previously DRR), Hyderabad named as IET 23832 having high Zn (19.50 ppm). Black glutinous type of cultivars is reported as an important source of oryzanol including antioxidant activity than normal rice (Pitija et al. 2013).

6.4 Phenotyping of Nutritional Traits

Easy qualitative method like Perl's Prussian Blue for Fe and DTZ staining method for Zn has been developed for estimation to conduct the preliminary selection procedure for genotype identification (Bhattacharjee et al. 2008). Fine-tuned optical-based quantitation method for Fe and Zn has been standardized through inductively coupled plasma-optical emission spectrophotometry (ICPOES) or widely used instrument for various element, atomic absorption spectroscopy (AAS) (Choi et al. 2007). Zn and Fe phenotyping in brown rice for about 126 lines has been estimated with destructive sampling (Anuradha et al. 2012). Lipid content of rice can be phenotyped using near-infrared spectroscopy (Qin et al. 2010). Sophisticated technology like electrospray ionization-mass spectrometry and various liquid chromatographies have been utilized for phenotyping of γ-oryzanol like bioactive compound from grain (Kim et al. 2013a, b).

7 Inheritance of Traits

Gamma oryzanol is controlled in a similar way a quantitative trait like yield is being controlled and varies in different environments (Bergman and Xu 2003). Outcome from classical breeding effort is in low pace as of unclear inheritance nature and the complex effect of environment on nutritional traits specifically for protein biomolecule (Coffman and Juliano 1987), though in some study inheritance patterns revealed as more complex for the protein content and amino acid makeup in *indica* rice and heritability was high and varies among cultivars (Chai et al. 1995). Protein content is not solely controlled by QTLs as different other genetic factors and interaction effects were identified in rice for nutrition rich breeding (Tan et al. 2001). Previous research findings of few aromatic rice line having higher Zn content established an indirect selection strategy for higher Zn and Fe content through aroma, but pleotropic effect was established, and linkage was also too weak studied in a F2-derived populations (Graham et al. 2004). Positive correlations among Fe and Zn level in different genotypes of bean are identified by CIAT researchers, where this co-segregation inheritance can be attributed to simultaneous selection of both the major nutritional factors. Genetic analysis showed the complex pattern of genetic advance of the higher Fe content character. Though the influence of the environment is fewer for expression of the trait, both additive and dominance gene actions contributed significantly. Hence, for breeding goal towards more Fe concentration in the rice grain, selection at early segregating generation need to avoid due to presence of some dominance effect (unpredictable/unfixable genes) and it should start from F5 onwards for that particular trait, parallaly, bulking can be done for other agronomic traits. Nutritional trait like Fe content has a reciprocal effect in some study which brings a selection of male or female parent to avoid less improvement (Gregorio et al. 1999). In other different studies regarding influence of endosperm, maternal effect towards grain protein and amino acid content has been identified, different from normal inheritance pattern (Shi et al. 2000).

8 Procedure of QTL Mapping

Different programmes like 'IM-ADD', 'ICIM-ADD' and 'ICIM-EPI' have been utilised for main effect of additive QTL and trait specific interaction QTL in all the environment (Li et al. 2008; Zhang et al. 2012).

8.1 QTL Mapping: A Key Target for Rice Breeding

Detailed genetic dissection of rice brought a clear picture for rice breeding. From yield improvement to biotic and abiotic stress tolerance breeding involves QTL identification as most of these cases' traits are complex and genetic mechanism of traits are also different. It is reported that the phenotypic variation of many complex traits like nutritional factors are controlled by multiple quantitative trait loci (QTLs)

involves interaction among those QTLs and succeeding environment invests a detailed mechanism for breeding such traits (Zhu et al. 2008). In some study qtl has been localized near waxy gene (Aluko et al. 2004). Protein content has been linked through qtl in brown rice. A set of total 14 qtl has been identified for grain protein content including 3 qtl for multi environment (Chattopadhyay et al. 2019) because grain protein content is influenced by environment and hence MEQ is important. Two qtl like *qPC-1* and *qGPC1*.1 have been identified with more stable nature for inheritance. The QTLs present in rice which leads to identify genomic region inside these qtl for fine mapping grain protein content region are important one. Though several qtl are responsible for grain protein content of rice as normal distribution found in several study, several regulatory genes are also controlling the character (Kumar et al. 2011) that proves complex control of the character. Association of protein content with brown rice trough three qtl like *qPC1.1, qPC11.1* and *qPC11*.2 has been shown (Qin et al. 2009). Another 5 qtl, viz. *qPC-3 to* 6 and 10, for protein content developed (Yu et al. 2009) and for milled rice two consistent qtl has been also developed (Zhong et al. 2011). For mapping qtl for lipid content region three QTLs on chromosomes 1, 2 and 5 through doubled haploid (DH) population from a cross between two sub-groups and a RFLP linkage map has been developed and detected (Hu et al. 2004). A linkage map was developed initially for 56 SSR marker and 116 STS (Qin et al. 2008). Around 4 years of extensive experiment, an extant of phenotypic variations like 13.8% and 11.3% through *QLC5.1*, within the interval 5019-RM289 with a mean LOD of 5.10 followed by *qLC6.1*inthe interval 6011-RM19696 with LOD of 4.21, respectively, were detected (Qin et al. 2010). Among various traits after oryzanol and important minerals, it is necessary to breed for amino acid composition. To identify genomic location for amino acid composition in a set of 190 RILs, 17 amino acids have been mapped (Wang et al. 2008). Two QTL clustered between RM 472-RM104 and RM125-RM542 have been detected explaining phenotypic variation beyond 30 and 40%, respectively. Breeding for polyphenol content can be done in indirect way as antioxidant, flavonoids compounds and phenolic content all hold both the markers RM339 and RM316 as the common markers for in pericarp (Yafang et al. 2011; Shao et al. 2011), while two qtl have been identified for pericarp polyphenolic content (Bres-Patry et al. 2001). Two QTLs explaining major variations like 24.3 and 15.4% for phytate concentration have been located on chromosomes 5 and 12 with LOD score more than 3 in both the case. The majority of P in grain accumulates as phytate identified by James et al. (2007) through correlation study of phytate with inorganic phosphate as strength of correlation was very high. QTL associated with folate biofortification has been identified in mapping population like recombinant inbred line and back cross inbred line (Dong et al. 2014), but this QTL is not located near to the region containing folate biosynthesis which indicated further detailed mapping and mechanism will lead to find out the actual insight and markers that will be utilized for breeding folate in rice. Further 10 QTLs contributing a wide range of 5.3–25.81% phenotypic variations for five different mineral elements including Cu, Ca and Mn with other two common elements on six chromosomes ranging from 1 to 9 linkage group have been identified (Lu et al. 2008).

8.2 Bioinformatics Tools for Identification of Genes for Nutrition Content

Gene harvesting from QTL region through bioinformatics analysis for identification of key genes for rice grain glutelin, globulin, prolamin and albumin can be analysed from rice annotation project database (Sakai et al. 2013) and oryzabase (Kurata and Yamazaki 2006) with the information regarding physical position.

8.3 Mapping Population

Using a DHs population James et al., identified three QTLs, within chromosomes 2, 8 and 12 controlling Fe content with 14–18% variation explained. RIL-based 14 QTL has been identified for two major target nutrition element from unpolished rice derived from population between 'Madhukar' and 'Swarna' (Anuradha et al. 2012). Another doubled haploid population derived from indica type "IR64"and japonica type "Azucena" is exploited for the analysis of QTL related to rice quality amylase, fat, protein, etc. (Bao et al. 2002). In a backcross population, a BC1F5 dense nutrient-rich quality rice has been developed through widely used technology RADseq (Peng et al. 2016).

8.4 Advanced Tools for Genetic Diversity for Nutritional Traits

Genome-wide SNP identification and marker-trait association are nowadays strategy to exploit marker that is stable throughout generation evolutionarily (Zhao et al. 2011) from genotyping data of 413 wide diverse accessions of *O. sativa* explaining more than 40,000 SNP and phenotyped them for a set of 34 traits including grain quality parameters. Transcriptional analysis by new parallel sequencing brought out several differentially expressed genes involved in biosynthesis of nutritional quality parameters and including protein metabolism and storage protein translocation to grain for biofortification (Venu et al. 2011). A very large set of rice germplasm has been used for genome-wide association mapping (Varshney et al. 2014), high-throughput genotyping platforms and re-sequencing where a similar approach has been used for 429 chickpea accessions from 45 countries to dissect diversity (Varshney et al. 2019). Genome-guided RNA-seq (Badoni et al. 2016), fine mapping (Zhang et al. 2013), genomics approaches, sequencing-by-synthesis (SBS) (Sun et al. 2015) and next-generation platforms for sequencing (Matsumoto et al. 2016) have been utilized recently for different traits but that can be used for nutritional traits improvement.

8.5 G X Environment Interaction

Ranking of genotypes at one or more environments determines breeder's selection strategy. For practical reasons to make ease of other statistical comparisons among genotypes may be ignored (Cramer and Beversdorf 1984). Ultimately, the impact of G × E interaction on the ranking of breeding lines at different environments is of interest.

8.6 Candidate Genes for Nutritional Traits

Rice seed protein, composed more than 60% glutelin and 20% prolamin, involves a complex mechanism with several genes (Kawakatsu et al. 2008; Xu and Messing 2009) controlling this trait. Most of the nutrient, stored in grain, depends on transporter protein and recently several transporter characterization, cloning and expression have been analysed (Table 1). A transgenic fingermillet overexpressing OsZIP1 transporter controlled by constitutive 35S promoter and endosperm-specific Bx17 for high Zn accumulation has been developed. In advanced overexpressed T1and T2 seeds, Zn content showed 10–15 mg/kg and 20 mg/kg respectively, higher than wild type (Ramegowda et al. 2013). Several studies identified associated gene that increase Fe and Zn level for biofortification in rice grain, obtained from engineering NAS genes or influencing other nutrient translocation gene-related pathway (Mahender et al. 2001). For lysine enrichment in rice grains, two genes like a separate kinase and dihydro picolinate synthase can be utilized through overexpressing as lysine insensitive form (Galili et al. 2002). Another mutant *Escherichia coli lysC* increased lysine content fivefold in canola and soybean and can be implemented in rice in a realistic way (Falco et al. 1995; Mazur et al. 1999). RLRH1 and RLRH2, two lysine-rich indigenous histone proteins, have been overexpressed to develop lysine biofortified rice (Wong et al. 2015). Genetic dissection of colour contributing to black rice pericarp for investigating polyphenolic compound two loci *Pb* and *Pp* located on chromosomes 4 and 1, respectively, has been identified (Yoshimura et al. 1997). Genetic factor, responsible for purple pericarp, *Pb* on chromosome 4, generated through deletion mutation within exon of *Ra* (Wang and Shu 2007). A transgene for a heat-stable phytase from *Aspergillus fumigates* has been characterized which increased the level of phytase by 130-fold more than wild (Lucca et al. 2001). To increase heat stability of phytase, codon optimization was done by Pasamontes et al. (1997).

9 Biotechnological Approach for Improvement

Alternative approach like genetic engineering to enhance nutritional values has been considered to be the potential tool for the sustainable delivery of nutrient-rich crops (Dias and Ortiz 2012). Biotechnological methods for lysine-rich rice breeding is to suppress the expression of lysine ketoglutarate reductase/saccharopine

Table 1 Genes for breeding nutritional traits in rice

Genes for nutritional factors	Functions	References
RLRH1, RLRH2	Lysine biofortification	Wong et al. (2015)
Granule-bound starch synthase I (GBSS I)	Determine amylose content	Tripathy et al. (2017)
Rc((basic helix-loop-helix protein)	Accumulation of proanthocyanidins	Sweeney et al. (2006)
Rd(dehydro flavonol reductase)	Anthocyanin and proanthocyanidins pathway	Furukawa et al. (2007)
OsZIP8a, OsZIP8c and OsZIP4b	Zn content	Gande et al. (2014)
OsYSL2, OsNAAT1 and OsNAC	High grain Zn content	Chandel et al. (2011)
OsYSL15	Fe transporters	Masuda et al. (2013)
Aspartate kinase (AK) and dihydrodipicolinate synthase (DHPS),	Lysine biosynthesis	Galili et al. (2002)
Lysine ketoglutarate reductase/ saccharopine dehydrogenase	Lysine degradation pathway, RNAi mediated mechanism can increase lysine	Zhu and Galili (2004), Hournard et al. (2007)
PURPLE PERICARP A, PURPLE PERICARP B	Pericarp pigmentation	Wang et al. (2009)
Black hull4 (Bh4)	Black hull colour of *O. Rufipogon*	Zhu et al. (2011)
Kala4	bHLH gene, black grain trait	Oikawa et al. (2015)
Anthranilate synthase alpha 1	Higher grain protein content	Chattopadhyay et al. (2019)
OsNRAMP1	Iron transport	Curie et al. (2000)
PG5a, RM1 and RP6	Were associated for proline	Chen et al. (2018)
Wx, AGPS2a	Was associated with Alb	Chen et al. (2018)
GluA1 and OsAAT2	Total seed storage protein	Chen et al. (2018)
OsAAP6 (amino acid permease,)	Controlling natural variation in GPC	Peng et al. (2014)

dehydrogenase (LKR/SDH), the key enzymes in the lysine degradation pathway, using antisense or RNA interference (RNAi) methods to maintain higher level of lysine (Zhu and Galili 2004). Improvement of rice protein and nutritional components through transgenic research is also paving the hope, like the expression of AmA1 seed albumin (Xu et al. 2017), overexpression of aspartate aminotransferase genes (Zhou et al. 2009) and the transfer of two artificially designed genes (Jiang et al. 2016). The first use of CRISPR/Cas9 system in rice nutritional improvements by knocking out five rice carotenoid catabolic genes (OsCYP97A4, OsDSM2, OsCCD4a, OsCCD4b and OsCCD7) increase β-carotene accumulation in rice endosperm (Yang et al. 2017). However, the targeted mutations in five carotenoid catabolism genes failed to boost carotenoid accumulation in rice seeds. Folate biosynthetic genes have been overexpressed in rice and caused a considerable increase in folate content in transgenic rice (Strobbe and Van Der straiten 2017).

An increase in γ-oryzanol content by transforming several genes involved in the terpinoid specifically isoprenoid pathway from daffodil and *Erwinia uredovara* into *indica*-type cultivars has been proposed by Hoa et al. (2003).

10 Golden Rice

To eradicate vitamin A deficiency Beyer et al. (2002) developed β-carotene rice through genetic engineering and providing this golden rice through farmers, particularly, for developing countries. For elimination of nutrition hunger in developing countries, golden rice (Ye et al. 2000) using different genes from distant source of transgene has been incorporated through transgenics including the genes *psy* (cloned from *Narcissus pseudonarcissus*) coding phytoene synthase (Schledz et al. 1996), *cryt1*(cloned from *Erwinia uredovora*) carotene desaturase (Misawa et al. 1993) and *lyc* coding lycopene cyclase that converts lycopene to β-carotene which have been introduced into the rice, driven by the endosperm-specific glutelin promoter (*Gt1*). The *crt1*was fused to the transit peptide (tp) sequence by the pea Rubisco small subunit (Misawa et al. 1993) to lead the accumulation of lycopene in the endosperm plastids. In recent experiment, it has been found that carotene-enriched staple food crop enhancing the bioavailability of nonheme Fe component (Garcia-Casal et al. 2000). Under the probable highest impact situation, it was estimated that the use of golden rice in India might save 1.38 million (disability adjusted life years) DALYs per year (Qaim 2010).

11 Conclusion and Future Prospect

In this chapter, we described the nature of rice grain, and its details need to improve towards nutrition dense and replacing the existing cultivar through advanced genomic approaches. Available genetic resources for breeding quality rice with nutritional factors have been mentioned that need to incorporate in other popular cultivars. In this situation, with limited information of QTL mapping of different traits except for rice grain protein content, several QTLs with higher phenotypic variation and stable over the environments can be used for further experiments. Research on genetic mechanism of element translocation and storage along with yield enhancement should be done simultaneously so that newly developed varieties are also nutritionally enriched. Nowadays to dissect overall genetic diversity and environmental interaction study involving diverse environmental pressure can identify behaviour of a complex trait. The Molecular breeding approach by identifying associated QTLs and isolating responsible alleles from the particular locus explains genetic mechanism because till now candidate gene-based breeding for particularly this trait has not been initiated at full pace. Instead, identification of QTLs, predicting putative gene that can explain a larger phenotypic variation can be advantageous for candidate gene-based approach. However, to improve rice nutritional quality to make it more nutrient dense, single gene by gene approach will not be attracting;

rather a genomic locus harbouring multiple QTL and controlling several nutritional facts that are being maintained evolutionarily year after year in some of the genetic resources must be incorporated. Involving newly identified genetic factors with additive effect and common pattern of inheritance, a simple breeding procedure can be applied. Complex genetic mechanism involves transgene introgression-oriented improvement beyond the close gene pool which lacks speedy translation procedure throughout the developing countries. If we need to erase hunger by a sensible, balanced nutrition with sufficient factors of growth and development, we need biotechnological tools to be utilized for rice breeding and improvement, particularly for quicker translation of QTL and gene discovery into high yielding varieties. Further success of nutrition rich rice development should fit snugly to some gaps like, as replacement of popular variety by improved variety breaking farmer's conventional choices and performance of that improved variety in different corners of the world including areas of nutrient-deficient soil to yield that nutrient dense crop yield and the level of bioavailability of nutrition. Plant breeding, alone, can't create a world with zero nutrition hunger, though breeding goals can reduce impact in the coming future. On the other hand, an alternative strategy for enriching nutritional status of rice bypassing breeding goals, involving spraying or soaking in vitamin solution and increasing retention efficiency, can lead to some sorts of recovery from nutrient deficiency.

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Genetic Enhancement of Nutritional Traits in Rice Grains Through Marker-Assisted Selection and Quantitative Trait Loci

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Abstract

Rice is a basic staple food of most countries including developed and less developed nations, and more importantly, 90% of population relies on rice across South Asian countries. Global climate change, increased urbanization, drought, and desertification have resulted in the significant drop in the rice production and also prompted researchers to develop novel varieties with increased productivity. Hence, novel varieties with enriched nutritional composition may offer wide benefits and could be a potential step in eradicating malnutrition among the less developed nations. According to the "International Rice Research Institute (IRRI)," 843 varieties have been developed until now, which have been generated by using both conventional and molecular breeding techniques. Genetic markers are forerunner in the present-day agriculture production system, which has anonymously contributed in the production of novel varieties with additional accuracy, reliability, and rapidity. Thus, it has significantly contributed in

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precision plant breeding, and dependency on molecular techniques such as marker-assisted selection and quantitative trait loci is inevitable for enhanced crop productivity in the near future. In the present chapter, an elaborate case study involving recent developments in enhancing the nutritional quality of rice crop using marker-assisted selection and quantitative trait loci has been represented.

Keywords

Rice improvement · Nutritional traits · Grain quality · Marker-assisted selection · Quantitative trait loci

1 Introduction

In the current scenario, quality of grains and its nutritional value has become a prime interest for producers as well as consumers. About 49 nutrients are commonly required for the development and normal growth, and this nutrient demand is globally fulfilled by cereals, specifically rice (Welch and Graham 2004). Among all these micronutrients, minerals play very important role in the human metabolism. Rice crop has relatively low content of some essential mineral elements such as zinc (Zn), calcium (Ca), and iron (Fe) in comparison to other main crops including maize, wheat, tubers, and legumes (Adeyeye et al. 2000). The widespread occurrence of osteoporosis and anemia was surveyed mostly in developing and some developed countries, and it has been found to be due to insufficient amount of Ca and Fe, respectively (Welch and Graham 1999). The developed nations are dealing with deficiency, thereby implementing food fortification management systems, but the same modes could not be affordable for developing countries. Therefore, alternative and affordable strategies to enhance the nutritional quality of rice have been reoriented to grow high-yielding varieties with nutrient-rich cultivars by adapting appropriate marker-assisted breeding or genetic approaches (Gearing 2015). In this modern crop breeding era, genomics is a vital tool to grow more proficient nutritional rice plants to reduce the consumers' health problems associated with mineral elements (Perez-de-Castro et al. 2012).

2 Enhancement of Mineral Accumulation Using Marker-Assisted Selection

The availability and development of dense genetic maps and an assembly of molecular genetic markers in rice plants have made the application of marker-assisted selection (MAS) possible for the traits controlled by major genomic regions or quantitative trait loci (QTL) (Choudhary et al. 2008). QTL mapping offers opportunities for recognition of identified genes associated with required traits by aligning genetic information with phenotypes. The genomic basis of accumulation of mineral nutrients in rice grains, identification of genes, and mapping of QTL provide

the source for formulating the strategies to improve the mineral contents in rice (Zhang et al. 2004). Several scientists have reported the genetic diversities of mineral contents in rice crops (Gregorio et al. 2000; Zhang et al. 2004; Anandan et al. 2011; Jagadeesh et al. 2013; Ravindra Babu 2013). However, to date, major considerations have been given for the development of genetically modified rice crops that contain high bioavailable elements particularly Fe and Zn. Likewise, the Indian Institute of Rice Research has also developed the genotype IET 23832 with increased Zn contents (19.50 ppm). Although brown rice has more content of Zn and Fe, around 70% of mineral contents are removed during polishing process because of their presence on the outer covering of kernel (Sellappan et al. 2009). Martinez et al. (2010) have found 20–25 ppm of Zn and 10–11 ppm of Fe contents in brown rice, while 16–17 ppm of Zn and 2–3 ppm of Fe in the milled rice.

2.1 QTLs for Mineral Elements in Rice

Quite a lot of QTLs have been defined in rice related to micronutrient quality traits with diverse genetic roots of interspecific and intraspecific crosses using selected molecular markers. Gregorio et al. (2000) observed three loci on chromosomes 7–9 that showed 19–30% difference for Fe contents in rice. Stangoulis et al. (2007) found a QTL for Fe on chromosome 2 showing 16.5% of phenotypic variance (PV) from double-haploid (DH) rice lines derived from Azucena and IR64. Further, an important Fe linked QTL close to RM6641 marker on chromosome 2 was observed by Garcia-Oliveira et al. (2009) from introgression population derived from *O. rufipogon* and Teqing. *O. rufipogon*, a wild rice variety, overall presents 26 QTLs for most favorable alleles, and chromosomes including 1, 9, and 12 hold 14 QTLs that is 45% of these traits. One major influence of QTL reported for largest section of PV 11–19% for Zn content was identified on chromosome 8 close to RM152 sequence repeat marker. James et al. (2007) used DH lines for three QTLs associated with Fe contents on chromosomes including 2, 8, and 12 with a total PV from 14 to 18%. They also identified two Zn linked QTLs on chromosomes 1 and 12 describing PV of 13–15%. Major effect of ten QTLs reported for Zn, copper (Cu), Ca, Fe, and manganese (Mn) micronutrients, and qFe-1 mineral trait has shown high PV with 25.81% as well as 7.66 LOD scoring (Norton et al. 2010). A total of 14 QTLs linked with Zn and Fe were identified from the unpolished rice grains of Madhukar and Swarna recombinant inbred lines (RILs) by Anuradha et al. (2012). Additionally, 24 Zn-associated genetic markers and 4 linked genes, namely, OsZIP8a, OsNAC, OsZIP4b, and OsZIP8c, with PV ranging from 4.5 to 19.0% were identified by Gande et al. (2014). Garcia-Oliveira et al. (2009) reported 31 significant QTLs for micronutrients Zn, Fe, Cu, and Mn and macronutrients such as magnesium (Mg), Ca, potassium (K), and phosphate (P) on all chromosomes except chromosome 7. Among total putative QTLs, chromosomes 1 and 9 showed maximum QTLs containing five QTLs for each. Earlier studies reported many QTLs for mineral nutrients linked with several chromosomal regions. QTLs for P on chromosomes 1 and 12 (Ni et al. 1998; Wissuwa et al. 1998; Ming et al. 2001;

Wissuwa and Ae 2001a, b), K on chromosomes 1 and 4 (Wu et al. 1998), and then Mn on chromosome 10 (Wang et al. 2002) have also been reported. Ten QTLs on 7 chromosomes associated with Zn, Fe, Ca, and Mn accumulation in rice grains have been observed by Lu et al. (2008). Zhang et al. (2014) identified a total of 134 QTLs for 16 microelements in unmilled rice, and among all QTLs, 6 were strongly linked and validated (Table 1, Fig. 1).

2.2 Transgenic Methods to Enhance the Mineral Accumulation in Rice Cultivars

Through transgenic methods, threefold enrichment of Fe contents has been observed in rice endosperm by introducing soybean ferritin gene by Goto et al. (1999). Similarly, Lucca et al. (2001) transferred the ferritin gene of common beans into rice grains that showed twofold enhancement of Fe content than control. Vasconcelos et al. (2003) also introduced ferritin gene of soybean into rice grains and observed twofold increase of Fe contents in the rough rice and threefold in the milled rice. Likewise, enhancement of Fe contents in the T1 brown rice seeds and T2 polish seeds was observed by Khalekuzzaman et al. (2006) as compared to reference lines. So Fe element concentration enhanced more than twofold in genetically engineered lines. Therefore, several researchers have made an attempt to enhance Fe elements in endosperm of rice by overexpressing the candidate genes responsible for Fe uptake from soil and involve in translocation from the shoot, roots and flag leaves to grains and by improving the proficiency of Fe accumulating proteins (Kobayashi and Nishizawa 2012; Lee et al. 2012; Bashir et al. 2013; Masuda et al. 2013; Slamet-Loedin et al. 2015). Many reports exhibited increase in Zn and Fe contents in rice by overexpressing endosperm-specific endogenous nicotianamine synthase gene (NAS) or other associated transporters genes. Masuda et al. (2009) introduced *Hordeum vulgare* NAS gene to rice grains and observed the significant increase of target trait with twofold to threefold higher accumulation of Zn and Fe in milled rice. Similarly, Zheng et al. (2010) reported increase in fivefold of Fe contents in polished rice through overexpression of NAS gene. Moreover, three homologous proteins of NAS, namely OsNAS1, OsNAS2, and OsNAS3, were overexpressed to enhance the twofold accumulation of Zn and Fe in the polished rice (Johnson et al. 2011). Lee et al. (2009) also reported that introduction of OsNAS3-D1 enhances the accumulation of Zn, Fe, and Cu from 1.7-fold to 2.9-fold in rice as compared to other wild-type grains at the seedling stage. Introduction of multiple genes such as barley IDS3 genomic regions and NAS gene overexpression, OsSUT1 promoter drive OsYSL2, and ferritin gene under endosperm-specific promoter control showed significant enhancement of Fe accumulation ranging 1.4–6-fold compared to milled rice seeds (Masuda et al. 2013). These results suggested that multiple-gene targeting strategy would provide more assistance for the enhancement of mineral accumulation in rice grains.

Table 1 QTLs associated with nutrient traits in rice population[a]

Chr	QTLs	Markers	Type	Grain traits	Populations	References
1	*qPr1*	RM493-RM562	RILs	PC	Zhenshan97B/Delong 208	Zhong et al. (2011)
	qPC1.1	1008-RM575	DHs	PC	Samgang/Nagdong	Qin et al. (2009)
	qP.1	RM3411	LT/TL-RILs	MAC-P	Teqing/Lemont	Zhang et al. (2014)
	qK.1	RM5501	LT/TL-RILs	MAC-K	Lemont/Teqing	Zhang et al. (2014)
	qPC1	RM472-RM104	RILs	PC	Zhenshan97/Nanyangzhan	Peng et al. (2014)
	qAa1	RM493-RM562	RILs	AAC	Zhenshan97B/Delong 208	Zhong et al. (2011)
	qP.1	RM495	LT/TL-RILs	MAC-P	Lemont/Teqing	Zhang et al. (2014)
	qCd.1	RM6840	LT-RILs	MAC-Cd	–	Mahender et al. (2016)
	qZn.1	RM34-RM237	DHs	Zn	IR64/Azucena	James et al. (2007)
	qMn.1	RM243-RM312	DHs	Mn	–	Mahender et al. (2016)
	qCo.1	RM490	LT/TL-RILs	MAC-Co	Lemont/Teqing	Zhang et al. (2014)
	qCa1–1	RM6480	ILs	MAC-Ca	*O. rufipogon*/Teqing	Garcia-Oliveira et al. (2009)
	qP1–1	RM212	ILs	MAC-P	–	Mahender et al. (2016)
	qFe1.1	RM243-RM488	RILs	Fe	Madhukar/Swarna	Anuradha et al. (2012)
	qFe1.2	RM488-RM490	RILs	Fe	–	Mahender et al. (2016)
	qAA.1	RM472-RM104	RILs	AAC-Asp/Thr/Glu/Gly/Ala/Cys/Tyr/Pro/EAA/Total	Zhenshan97/Nanyangzhan	Wang et al. (2008b)
	qFe.1	RM259-RM243	RILs	Fe	Zhenshan 97/Minghui 63	Kaiyang et al. (2008)
2	*qFe2–1*	RM6641	ILs	MIC-Fe	*O. rufipogon*/Teqing	Garcia-Oliveira et al. (2009)
	qPC-2	RM5897-RM6247	RILs	PC	Chuan7/Nanyanghan	Lou et al. (2009)
	qCu.2	RM6378	LT/TL-RILs	MIC-Cu	Lemont/Teqing	Zhang et al. (2014)
	qSr.2	RM3688	LT-RILs	MAC-Sr	–	Mahender et al. (2016)
	qFe.2	RM53-RM300	DHs	Fe	IR64/Azucena	James et al. (2007)
	qAA.2	RM324-RM301	RILs	AAC-His	Zhenshan97/Nanyangzhan	Wang et al. (2008b)
	qAA.2	RM322-RM521	RILs	AAC-Val/Ile/Leu/His/Phe	–	Mahender et al. (2016)
	qLip-2	RM5619-RM1211	DHs	PC	Cheongcheong/Nagdong	Yun et al. (2014)

(continued)

Table 1 (continued)

Chr	QTLs	Markers	Type	Grain traits	Populations	References
	qPro-2	RM12532-RM555	DHs	PC	Cheongcheong/Nagdong	Lee et al. (2014)
	qFe.2	RM452	LT/TL-RILs	MIC-Fe	Lemont/Teqing	Zhang et al. (2014)
	qMn2–1	RM6367	ILs	MIC-Mn	*O. rufipogon*/Teqing	Garcia-Oliveira et al. (2009)
	qS.2	RM266	LT-RILs	MAC-S	Lemont/Teqing	Zhang et al. (2014)
3	*qCa.3*	RM5626-RM16	LT/TL-RILs	MAC-Ca	–	Mahender et al. (2016)
	qRb.3	RM489	LT-RILs	MAC-Rb	–	Mahender et al. (2016)
	qAA.3	RM520-RM468	RILs	AAC-Tyr	Zhenshan97/Nanyangzhan	Wang et al. (2008b)
	qMg3–1	RM5488	ILs	MAC-Mg	O. *rufipogon*/Teqing	Garcia-Oliveira et al. (2009)
	qCa.3.	RM200-RM227	RILs	Ca	Zhenshan 97/Minghui 63	Kaiyang et al. (2008)
	qPC-3	RM251-RM282	RILs	PC	Xieqingzao B/Milyang	Yu et al. (2009)
	qZn3.1	RM7-RM517	RILs	Zn	Madhukar × Swarna	Anuradha et al. (2012)
	qPC-3	RM251-RM282	RILs	PC	Xieqingzao B/Milyang	Yu et al. (2009)
	qMn.3	RM227-R1925	RILs	Mn	Zhenshan 97/Minghui 63	Kaiyang et al. (2008)
	qCu.1	R1925-RM148	RILs	Cu	–	Mahender et al. (2016)
4	*qAA.4*	RM348-RM131	RILs	AAC-Thr/Gly/His/Arg	Zhenshan97/Nanyangzhan	Wang et al. (2008b)
	qcpb4	E12M61.256	RILs	CPB	Cypress/Panda	Kepiro et al. (2008)
	qcph4	E12M61.256	RILs	CPH	–	Mahender et al. (2016)
5	*qCu.5*	C1447-RM31	RILs	Cu	Zhenshan 97/Minghui 63	Kaiyang et al. (2008)
	qPA.5	RM305-RM178	DHs	PA	IR64/Azucena	James et al. (2007)
	qFC-5	RG480-RM274	RILs	FC	Xieqingzao B/Milyang	Yu et al. (2009)
	qFe5.1	RM574-RM122	RILs	Fe	Madhukar/Swarna	Anuradha et al. (2012)
	qCa5–1	RM598	ILs	MAC-Ca	*O. rufipogon*/Teqing	Garcia-Oliveira et al. (2009)
		RM421	LT/TL-RILs	MIC-Zn	Lemont/Teqing	Zhang et al. (2014)
6	*qLlp-6*	RM586-RM1163	DHs	LC	Cheongcheong/Nagdong	Yun et al. (2014)
	qPC-6	RM190-RZ516	RILs	PC	Xieqingzao B/Milyang	Yu et al. (2009)

	qFC-6	RM190-RZ516	RILs	FC	Xieqingzao B/Milyang	Yu et al. (2009)
	qCu6–1	RM204	ILs	MIC-Cu	*O. rufipogon*/Teqing	Garcia-Oliveira et al. (2009)
	qZn.6	RZ398-RM204	RILs	Zn	Zhenshan 97/Minghui 63	Kaiyang et al. (2008)
	qPC-6	RM190-RZ516	RILs	PC	Xieqingzao B/Milyang	Yu et al. (2009)
	qMg.6	OSR 21	LT/TL-RILs	MAC-Mg	Lemont/Teqing	Zhang et al. (2014)
7	*qPc7*	RM270-C751	DHs	PC	Yuefu/IRAT109	Yongmei et al. (2007)
	qMn.7	RM214	LT/TL-RILs	MIC-Mn	Lemont/Teqing	Zhang et al. (2014)
	qAA.7	RM125-RM214	RILs	AAC-Pro/Gly/Met/Arg	Zhenshan97/Nanyangzhan	Wang et al. (2008b)
	qZn7.3	RM501-OsZip2	RILs	Zn	Madhukar/Swarna	Anuradha et al. (2012)
	qFe7.1	RM234-RM248	RILs	Fe	–	Mahender et al. (2016)
	qP.7	RM70-RM172	DHs	MAC-P	IR64/Azucena	James et al. (2007)
	qPC.1	R1245-RM234	RILs	PC	Zhenshan97/Minghui 63	Tan et al. (2001)
	qPr7	RM445-RM418	RILs	PC	Zhenshan97B/Delong 208	Zhong et al. (2011)
8	*qZn8–1*	RM152	ILs	MIC-Zn	*O. rufipogon*/Teqing	Garcia-Oliveira et al. (2009)
	qAA.8	RM137-RM556	RILs	AAC-Tyr	Zhenshan97/Nanyangzhan	Wang et al. (2008b)
	qAA.8	RM447-RM458	RILs	AAC-Cys	–	Mahender et al. (2016)
	qK8–1	RM3572	ILs	MAC-K	*O. rufipogon*/Teqing	Garcia-Oliveira et al. (2009)
	qZn.8	RM25-R1629	RILs	Zn	Zhenshan 97/Minghui 63	Kaiyang et al. (2008)
	qCu.8	RM201-C472	RILs	Cu	–	Mahender et al. (2016)
	qFe.8	RM137-RM325A	DHs	Fe	IR64/Azucena	James et al. (2007)
9	*qAa9*	RM328-RM107	RILs	AAC	Zhenshan97B/Delong 208	Zhong et al. (2011)
	qP9–1	RM201	ILs	MAC-P	*O. rufipogon*/Teqing	Garcia-Oliveira et al. (2009)
10	*qMg.10*	RM467	LT-RILs	MAC-Mg	Lemont/Teqing	Zhang et al. (2014)
	qAA.10	RM467-RM271	RILs	AAC-Cys/Leu/Ile/Phe	Zhenshan97/Nanyangzhan	Wang et al. (2008b)
	qPC-10	RM184-RM3229B	RILs	PC	Xieqingzao B/Milyang	Yu et al. (2009)
	qPro-10	RM24934-RM25128	DHs	PC	Cheongcheong/Nagdong	Yun et al. (2014)
11	*qMg.11*	RM332	LT/TL-RILs	MAC-Mg	Lemont/Teqing	Zhang et al. (2014)

(continued)

Table 1 (continued)

Chr	QTLs	Markers	Type	Grain traits	Populations	References
	qCu.11	RM167	LT-RILs	MIC-Cu	–	Mahender et al. (2016)
	qPC1.11	1027-RM287	DHs	PC	Samgang and Nagdong	Qin et al. (2009)
	qFe.11	RZ536-TEL3	RILs	Fe	Zhenshan 97/Minghui 63	Kaiyang et al. (2008)
	qPC1.11	RM287-RM26755	DHs	PC	Samgang and Nagdong	Qin et al. (2009)
12	*qPA.12*	RM247-RM179	DHs	PA	IR64/Azucena	James et al. (2007)
	qFe.12	RM270-RM17	DHs	Fe	–	Mahender et al. (2016)
	qZn.12	RM235-RM17	DHs	Zn	–	Mahender et al. (2016)
	qFe12.2	RM260-RM7102	RILs	Fe	Madhukar/Swarna	Anuradha et al. (2012)
	qFe12.1	RM17-RM260	RILs	Fe	–	Mahender et al. (2016)
	qZn12.2	RM260-RM7102	RILs	Zn	–	Mahender et al. (2016)

AAC amino acid content, *Ala* alanine, *Arg* arginine, *Asp* aspartic acid, *Ca* calcium, *Cd* cadmium, *Chr* chromosome, *Co* cobalt, *CPB* crude protein brown rice, *CPH* crude protein head rice, *Cu* copper, *Cys* cysteine, *DH* double haploid, *EAA* essential amino acids, *FC* fat content, *Fe* iron, *Glu* glutamic acid, *Gly* glycine, *His* histidine, *Ile* isoleucine, *ILs* introgression lines, *K* potassium, *LC* lipid content, *Leu* leucine, *MAC* macroelement, *Met* methionine, *Mg* magnesium, *MIC* microelement, *Mn* manganese, *NF* nutrition factors, *P* phosphorus, *PA* phytic acid, *PC* protein content, *Phe* phenylalanine, *Pro* proline, *RB* rice bran (%), *RILs* recombinant inbred lines, *S* sulfur, *Sr* strontium, *Thr* threonine, *Tyr* tryptophan, *Val* valine, *Zn* zinc

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3 QTL Detection and Application for Improving Nutrients in Rice

Rice is a major source of Fe, Ca, energy, thiamine (vitamin B1), riboflavin (vitamin B2), niacin, and protein in the diet (Rohman et al. 2014). The calorie of the rice grain is similar to wheat and the protein content is lower than wheat (Saunders 1990). Rice has less protein compared to meat and legumes, but the sugar and starch in it are higher than potatoes, legumes, and wheat. Few molecular studies have been performed on the nutritional properties of rice, but many activities have been performed in the field of molecular transformation with the help of genetic engineering to improve the nutritional properties of rice.

The amount of fatty acids affects the nutritional value and stability of rice storage. Apparently, more than 48 QTLs have been identified for the amount of rice fatty acids. Chromosomes 1, 3, and 6 have the highest number of QTLs for this trait. Wang et al. (2008a) showed that QTLs related to fat content (FC) are actively expressed during the grain filling process. In their study, 11 unconditional QTLs and 10 conditional QTLs have been found, most of which were expressed in the early stages of the growth and development. Their results demonstrated that fat accumulation is controlled by genes that are time-dependent. Liu et al. (2009) identified 14 QTLs for the amount of fat that were located on chromosomes 1, 3, 5, 6, 7, 8, and 9. *qCFC5* is a major-effect QTL which is located on chromosome 5 and is contributed in crude FC improvement. Ying et al. (2012) identified 29 QTLs for the composition of fatty acids that were distributed on all chromosomes. Shen et al. (2012) identified two QTLs for the FC on chromosome 7 that were consistently expressed. These two QTLs were identified in all three environments and later identified in the other six environments with the help of hybridization. Their results showed that steady-state and main-effect QTLs are suitable options for FC improvement through MAS programs (Table 1, Fig. 1).

The protein content of rice grains is superior to corn, wheat, and sorghum in terms of lysine (Zhang et al. 2008), and it has more balanced amino acids. High-protein rice has a high potential to improve human nutrition. Optimization of protein compounds and essential amino acids in combination with other biochemical properties can have a significant impact on the nutrition of the world's population. Protein deficiency is of great importance in terms of quantity and quality (volume of essential amino acids) in the diet. Improving the protein reserves in rice is a major goal of improving its nutritional quality and quantity. Grain storage proteins can be divided into albumin, glutelin, prolamin, and globulin. Glutelin, as the major protein of rice grain, comprises at least 81% of the total grain protein. Useful reports have been published on QTL mapping of protein content. In total, more than 50 QTLs have been identified for grain protein content. These QTLs have covered all 12 chromosomes. Chromosomes 1, 2, and 7 have more QTLs than other chromosomes. In this regard, Zhang et al. (2008) identified major-effect QTLs for globulin, albumin, glutelin, and prolamin content, respectively. QTLs that affect the number of different protein components are most likely located on the same locus. In another study, Wang et al. (2008b) identified 18 QTLs for different amino acids.

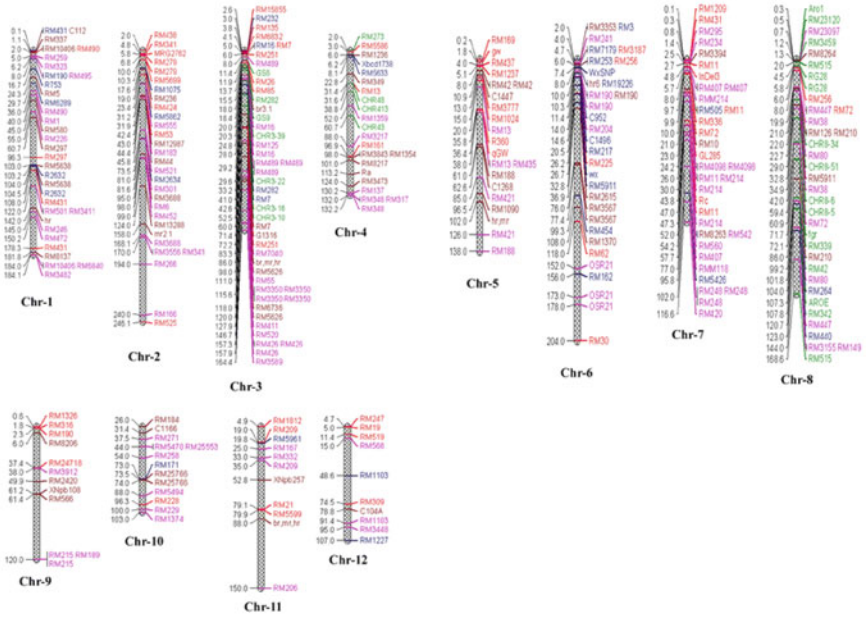

Fig. 1 Rice grain nutritional quality-associated molecular markers (on right) and their positions (cM, left side) on 12 chromosomes. *CP* cooking properties (blue), *FRG* fragrance of rice grain (green), *GA* grain appearance (red), *MPGQ* milling properties of grain quality, *NF* nutrition factors (pink) [Reproduced from Mahender et al. (2016), under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/)]

They found major-effect QTL at the end of chromosome 1 which is related to amino acid metabolism pathway. This locus consists of more than two separate QTLs (Wang et al. 2008b). Lu et al. (2009) identified 12 QTLs for the total amino acid content on five different chromosomes. These results are suitable for marker-assisted breeding programs and identifying candidate genes aimed at promoting rice amino acids.

The human body needs nutrients such as carbohydrates, oils, proteins, vitamins, trace elements, and minerals (selenium, Zn, Fe, Cu, and iodine) for growth and health (Welch and Graham 2004). The amount of Zn and Fe in cereals such as rice and maize is under the control of quantitative traits and reduces the speed of breeding programs for these traits (Long et al. 2004). Also, continuous changes in Fe and Zn concentration have made it a quantitative trait. At the moment, our knowledge about the genes controlling the homeostasis of mineral cations is poor. It has been suggested that the production of micronutrients and their accumulation in seeds are under genetic control (Genc et al. 2009). With the development of genomic analysis tools, many genes and gene loci involved in enhancing mineral content have been identified. Therefore, this knowledge can be very useful for improving crop efficiency, nutritional value, and food safety. Stangoulis et al. (2007) mapped QTLs tightly linked to total P, inorganic P, Fe, Zn, Cu, and Mg concentrations. Biradar

et al. (2007) mapped QTLs for Zn and silicon content in rice grains. Using the single-marker analysis for Zn content, six QTLs were located on chromosomes 1, 4, 5, 8, 9, and 11 with an $R^2 = 4.4–9.5\%$. Garcia-Oliveira et al. (2009) identified 31 possible QTLs for Mn, K, Zn, Fe, Cu, Mg, Ca, and P contents and introduced the superior lines for the mineral content. It is likely that myo-inositol 1,2,3,4,5,6-hexakisphosphate (phytic acid) forms complexes in rice grains with ions such as Ca, Zn, and Fe and reduces bioavailability of these minerals for humans. A set of mutated rice lines has been isolated with low phytic acid content that can increase the biological availability of minerals in rice (Liu et al. 2007). Norton et al. (2010) identified 41 QTLs related to the amount of major elements in the rice grain. Du et al. (2013) identified major-effect QTLs for the amount of Mn, Mg, Fe, Ca, Zn, and P in brown rice in two distinct environments of China. In this study, due to the small number of QTLs detected in the two environments, they found that the QTLs associated with the accumulation of minerals in rice grains are highly environmentally dependent. Some mutants have yielded some important markers (Tan et al. 2013; Zhao et al. 2008). These mutants and markers can help to produce new varieties of rice that have higher bioavailability of minerals (Table 1, Fig. 1).

Phytochemical properties such as phenol and flavonoids found in vegetables, fruits, and cereals, including rice, reduce the risk of cancer, vascular disease, and type 2 diabetes. These compounds help the antioxidant activity and fight against free radicals. Conventional breeding methods are one way of increasing the nutritional quality of rice and combat nutrient deficiencies. To improve phenol and flavonoid compounds and antioxidant capacity, the genetic structure of these traits must be known (Jin et al. 2009). Phenols are compounds that in their structure have one or more colorless or slightly pink crystalline rings with a specific odor associated with one or more hydroxyl groups (Ghasemi et al. 2019). Phenolic compounds are known as antioxidants. As loci controlling phenolics, flavonoids, and antioxidant capacity have been reported by Tan et al. (2001), Jin et al. (2009) showed that the number of antioxidant capacity, phenolic compounds, and flavonoids is controlled by three major-effect QTLs. In this study, the QTL on chromosome 2 had a common effect on the number of phenolic compounds and flavonoids. Shao et al. (2011) through simultaneous mapping of a variety of rice germplasm identified different QTLs for these traits. 4 and 6 QTLs were linked with the number of antioxidant capacity, phenolic compounds, and flavonoid, respectively. Of these, four QTLs controlling the content of phenolic compounds were common with the other two traits. Simultaneous mapping of different traits in 361 samples of white or no pigmented rice showed that the RM346 marker is linked with the amount of phenolic compounds.

4 Conclusion

Rice nutritional traits affect dietary values of grain, and they are essential to minimize malnutrition in developing countries. Rice breeding methods are rapidly getting advanced by exploiting transgenic aided breeding approaches to develop nutrient-rich rice varieties. The integration of QTLs and molecular marker in rice

grains will support breeders to assemble targeted genes for value-added rice production at commercial scale. Compatible QTLs using MAS could be integrated into single rice cultivar to formulate the required genotype with improved nutritional quality traits. The current knowledge on nutritional quality-related genes and recent advancement in genomic technologies could augment for refining the nutritional traits of rice for mankind.

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Breeding Approaches to Generate Biofortified Rice for Nutritional Enhancement

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Abstract

Biofortification is a process of improving plant's nutritional contents to overcome malnutrition. Approximately, half of the global population have vitamin A, iron and zinc deffeciency that leads to xeropthalmia, anemia an impaired immune function respectively. Peoples are counseled to be careful about the diversification of their routine diet in order to overcome the micronutrient deficiency problems. It's also a bitter reality that peoples can't afford food supplements due to their economic issues. Hence, in the above-said scenario, agronomic staple crops, i.e., wheat and rice, could be the best choice to solve this alarming issue. Staple crop biofortification have multiple advantages like rapid results, cost-effectiveness, accessibility, and handling and application of breeding and biotechnological techniques based on biofortification. Various agronomic and molecular approaches can be used for biofortification, namely, dose of nutrition, method of nutrient application, biofertilizer, sensitive stages for plant nutrition, zero tillage, plant growth promoters, microRNAs, epigenetics, genome engineering, genome editing, transformation via bioballistics, transformation via *A. tumefaciens*, patch clamp, mutant study, proteomics, protein expression, etc.

Keywords

Biofortification · Rice · Breeding · Crop diversification · Iron · Zinc · Vitamin A

1 Introductions

Rice is a major and essential food crop because it feeds almost half of the world's population as well as supports the economy (GRiSP, Global rice Science partnership 2013). In 2017, almost 90% of rice was consumed and produced in Asia (FAO 2017). In 2010, consumption of rice increased by 19.8% (388 million tons), and it is expected to continuously increase in 2035 to 465 million tons, due to the effects of different factors such as Westernization of Asian foods, urbanization, and growth revenues (GRiSP 2013; Kelly 2016). In addition, for the next 30 years, rice is believed to remain as a staple food in developing countries (Timmer et al. 2010). Literature has also demonstrated that in the metropolitan regions of Southeast Asia, especially in Indonesia, the Philippines, and Malaysia, rice is still the staple food for daily consumption (Lipoeto et al. 2013). Current Asian generations may consume less rice compared to their parents until Asians completely cease to consume rice (Timmer et al. 2010). On the other hand, rice consumption in West nations has increased (Ferrero 2004). Rice is a healthy food that's why, its consumption increases day by day (Suwannaporn and Linnemann 2008).

Indeed, consumers are inclined to buy high-quality rice especially those with increasing income (Timmer et al. 2010). Certain rice-consuming countries like Bangladesh and China shifted toward producing high-quality rice (Minten et al. 2011). Currently, consumers are distinguishing rice based on different parameters of nutrition and grain quality, for example, protein availability, texture, and fragrance

(Juliano 2006; Abdullahi et al. 2011). Furthermore, the units of protein, vitamins, and micronutrients also have significant nutritional values which help to control necessary nutrient deficits in rice-sustaining developing states (Arsenault et al. 2010; Pinkaew et al. 2013; Htet et al. 2016). Thus, rice does not simply an essential for filling the stomach every day, but its nutritional quality also fascinates the consumer's appetite and fulfils their nutritional demands.

The quality of rice cereal is assessed through a diversity of traits. The selectable quality of rice grain differs with area, culture, and people. The grain quality characteristics *i.e.* chalkiness, width, rupture, length and color that affects it market value beacuese of consumer's first choice (Tomlins et al. 2007). Seeds elongation, fragrances, thickness of gel, temperature of gelatinization, the protein and amylose content are the features of eating and cooking qualities (Champagne 2008). In addition, the price of fragrant rice in homegrown and export markets is greater than that of non-fragrant rice. Rice contains nutritive properties, i.e., micronutrients such as iron, zinc, and vitamin A (Brar et al. 2012).

Besides yield, rice grain and nutritional qualities must keep up with the burgeoning demand and changing lifestyle of consumers. Rice breeders have to tailor the rice grain and nutritional qualities to suit the preference of discerning consumers and also nourishing the people of their respective countries. Each nation has an outstanding rice production voyage, which generates rice varieties that its local growers and customers generally accept. Several high-quality rice traits demonstrate individual pride, culture, and high price on the national and global markets, such as the traits in Pakistan, India, and Bangladesh Basmati rice, in Thailand Khao Dawk Mali, in Iran Sadri, and in the Philippines Azucena (Garris et al. 2005; Calingacion et al. 2014; Ashfaq et al. 2015). These varieties have been produced by conventional breeders, and nowadays, some of the parent varieties are used in breeding.

The progress in gene editing, DNA markers and sequencing techniques include practical tools for plant breeders to enhance features of rice plant that were not possible with traditional reproduction methodologies. Markers of DNA have been included in the crop variety and breeding approaches in relation to the phenotype selection and assessment. After the development of sequencing of DNA, genome of rice has completely sequenced which makes gene annotation easier (IRGSP 2005). The updated RAP-DB (Rice Annotation Project Database) data unlocks the opportunities to improve its genetic engineering by genomic studies (Sakai et al. 2013). In addition, advances in gene editing of nucleases in other plants would offer a substitute technique for enhancing rice quality for rice breeders. Such latest developments would therefore help rice producers to enhance rice grain and dietary characteristics.

1.1 Zinc Deficiency Scenario

World's agricultural soils are 50% deficient with Zn and identified as the most critical micronutrient deficiency in crop globally. Approximately two billion

individuals around the world are affected by Zn deficiency, and about 1.5 million children die of Zn starvation every year. Overall, around 0.8 million individuals are at annual risk of death, and close to 0.45 million infants are at threat every year from zinc deficiency (WHO 2019). India has one of the largest levels of Zn soil and human diet deficiencies. 50% of Indian land is Zn-deficient, and if there is no management performed, it is expected to increase up to 63% by 2025. In India, every year, approximately 0.15 million kids die from shortage of Zn. Deficiency of zinc is linked to 25% of infant mortalities worldwide, i.e., below 5 years of age with diarrhea (IZA 2014).

1.2 Vitamin A Deficiency (VAD)

Deficiency of vitamin A is a root cause of xerophthalmia and night blindness. Xerophthalmia is the shortage of vitamin A concentrations in blood serum (greater than 0.35 μmol/l) (Hotz and McClafferty 2007). It's an eye-opener scenario that almost 250 million preschool children are deficient in vitamin A. Furthermore, 2.5 to 5 million children become blind every year due to VAD whereas half of them dying within 12 months after losing their sight (WHO 2019).

1.3 Iron Deficiency Anemia (IDA)

IDA accounts for 30% of worldwide anemia. This is a widespread issue of global health influencing both developed and developing countries. It is more common in pregnant females and 40% preschool children. IDA is capable of causing maternal hemorrhage with 20% of all maternal deaths involved. Major IDA risk variables include (1) low dietary consumption of Fe and poor absorption of iron, (2) food conssiting of high in phenolic or phytate compounds, and (3) stages of life such as pregnancy and growth when particularly Fe is required (WHO 2019).

1.4 Nutrition Gap

Daily consumption of 600 μg, 15 mg, and 15 mg of vitamin A, iron, and zinc, respectively, is recommended (Allowances 1989). In India, Swarna is the most commonly cultivated and consumed variety of rice that makes up to 0.78 mg Fe/100 g of white rice and 2.28 mg Zn/100 g of brown rice. By taking rice/meal two or three times a day, a person can only get 2–3 mg iron and 7–8 mg zinc, 1/5 and 1/2 of the recommended daily consumption of Fe and Zn, respectively.

1.5 Approaches to relieve Zinc, vitamin-A and Iron deficiency

Micronutrient malnutrition issues can be overcome by following methods (Diana et al. 2017). They can be written as follows:

Change of Diet Everyone should know about diverse diets and their importance and not limit their diet to certain crops only, which could cause malnutrition and weaken their physiochemical and biochemical methods.

Supplementation Various packaged food is available in market supplemented with micronutrients like Zinc, Iron, Iodine etc. to boost human diet but they are costly, which rarely accessed by peoples with poor background.

Biofortification It is the improvement of nutrients mostly needed by people in food crops.

3 billion people have less than US$ 2 income per day whereas 1.5 billion peoples have less than US$ 1 per day. Hence, cannot afford a diversified diet or supplements. It costs just as little as US$ 0.73–7.31 if wheat and rice become biofortified to save a healthy life.

2 What Is Biofortification?

The growth of nutrient-dense essential food cultivars via the best conventional breeding practices and current biotechnology without sacrificing agricultural performance and acquiring of key consumer preferred features is biofortification. Biofortification aims at increasing the nutrition of plant foods. Nutrients are added to foods during their processing in conventional fortification (Nestel et al. 2006).

2.1 Options for Biofortification

Several agronomic and genetic possibilities exist for biofortification. whereas agronomic biofortification is also called ferti-fortification. According to Rajendra (2009), fertilizing crops that contain micronutrients are called ferti-fortification. It shows immediate results and is usually used for enhancing yield. Therefore, bio-fortification could be a source of reaching populations where supplementation and conventional fortification activities may be difficult to implement.

2.2 Reasons for Biofortification

Rice is a vital crop to more than one billion people. Rice endosperm has deficiency of several micronutrients such as proteins and vitamins. The aleurone layer of dehusked rice grain is rich in nutrients but is removed during polishing and grinding. Crude

rice is contaminated in flavor—it is unpleasant and smelly. Recommended rice provides 30–50% calories daily. For its increased malleability, rice acts as a significant role in food safety.

Some β-carotene enriched popular rice varieties are:

IR 64, IR 36: Superior varieties that have preliminary Asian traits
BRRI dhan 29: Commonly found *boro* rice variety of Bangladesh
PSB Rc 82: Prevalent rice variety of the Philippines
OS 6561: Prevalent in Vietnam
Chehirang: Chief variety being used in Indonesia
Swarna: Carries significance in India

3 Bioavailability

In a typical food, the quantity of nutrients which is functional and palatable by the users is referred to as bioavailability. In essential crops, absorption of iron concentration does not depend on the enhancement of iron concentration, because many crop cultivars have diminished or enhanced the absorption of iron under high concentration. Several aspects disturb the bioavailability of zinc and iron in crop such as physiological and nutritional position of people, postharvesting process, growth circumstances, and constituents of meal. Phytic acid inhibits the absorption of iron and zinc, but on the other hand, nicotianamine and ascorbic acid enhance the uptake of Fe (Zheng et al. 2010). Taking a goo combination of food may also prove helpful for humans. In adult, inclusion of guava in rice based food was exposed to improve bioavailability of nonheme iron, wheras uptake of zinc was not observed (Nair et al. 2013). This would be required to increase the simultaneous bioavailability of zinc and iron. Zn and Iron bioavailability in human beings can be monitored by analyzing the quantity of ingested labelled nutrients (radioisotopes/stable), after feeding test meals (Nair et al. 2013). However, due to employment charges and related costs, these systems cannot be used as selection tools. Since 1998, in order to evaluate the bioavailability of zinc, carotenoids, and iron from numerous refreshments and foods, different screening methods such as colon adenocarcinoma cells have been used (Nemirovsky et al. 2014). The model contains mediated intestinal and gastric digestion of a test sample with differentiated cultures of CaCO-2 cells. For bioavailability in CaCO-2 cells, absorption of zinc and development of ferritin revealed to digests zinc and iron containing are used as substitute for bioavailability.

Uptake of zinc and iron is inhibited by phytic acid although uptake of iron is increased by NA and vitamin C (Zheng et al. 2010). With phytic acid, combination of zinc and iron has significant and adverse effect on the uptake of these elements in living bodies because these are not degraded due to deficiency of phytase enzyme. Hence, bioavailability of zinc and iron is enhanced by minimizing the phytate. Mutants of barley and maize have been produced with up to 95% minimum level of phytate (Raboy 2002). Different methods would be adopted to decrease the

phytate level, but in biosynthesis of cereals, mutations are the efficient method (Holm et al. 2002). Efforts are being made to reduce the phytate level by transgenesis or expressing gene for phytase (Agarwal et al. 2018). In rice endosperm, increment of 130-fold of the phytase gene was exposed by *Aspergillus fumigatus*. In maize and soybean, ferritin gene and phytase gene were expressed and significantly enhanced up to 20–70% of phytase and iron (Drakakaki et al. 2005). About 3.85 downregulation of inositol phosphate kinases exposed to T4 transgenic seeds improved via RNAi-mediated seed-specific silencing. These rice endosperms stored about 1.8-fold Fe contents by decreasing the phytic acid levels (Ali et al. 2013). In transgenic wheat, phytase gene related to heat stable increased the bioavailability of zinc (Brinch-Pedersen et al. 2007).

If foods have suitable promoters of bioavailability of Fe e.g. vitamin C, it can be eliminated antinutrients effects on bioavailability of micronutrients (Ashmead and Christy 1985). Poor solubility of ferric iron, due to its hydrolytic polymerization followed by precipitation in aqueous solution at neutral pH, limits its absorption in intestinal cells. Enhancing the solubility of Fe^{3+} is compulsory for increasing its absorption, and this may be carried out either via chelation or by reduction. A critical increment of NA in rice grain was seen by overexpression of NAS gene in rice seeds. Zheng et al. (2010) revealed that above two-fold change, Fe bioavailability from high NA endosperm as compared to control, utilizing a Caco-2 cell model, and recommended NA to be an intense enhancer of bioavailability of zinc and iron. Zinc and iron bioavailability was also measured from recombinant hereditary appearances (Madhukar χ Swarna RILs) which have excessive zinc and iron contents. Moreover, within the sight of vitamin C, ferritin initiation was expanded twofold, utilizing Caco-2 cells, and Zn take-up expanded triple from RIL with higher zinc and iron contents contrasted with a famous genotype, Swarna (unpublished information).

4 Conventional Breeding Approaches

Breeding genotypes is the best alternative to minimize the deficiency of micronutrients due to increased vitamins and minerals. Earlier, increasing productivity of rice genotype with rich micronutrients was not focused upon (Graham and Welch 1996). Recent considerations include focusing on increasing the yield along with the quality of rice grains because micronutrients are very important for human health. Screening of germplasm of local cultivars, wild species, and conventional varieties can be used to control natural allelic differences in rice to generate varieties supplemented with micronutrients. This is usually the initial step in breeding database aimed to increase grain zinc and iron. Wild varieties of rice carried enormous genetic variation for both zinc and iron. Specific and modern varieties have been exchanged by traditional germplasms, with genetic loss occurring in wild varieties. Many germplasms that are stored in gene banks allow for significant pool of variation in genetics that leads to development (Gepts 2006; Jahan et al. 2013). Anuradha et al. (2012) noticed differing concentrations of iron and zinc in

126 varieties of brown rice genotype, with Fe (6.2–71.6 mg/kg) in concentration while zinc extending from 27 mg/kg to 67 mg/kg. Huge variations were described by Kumar et al. (2012) in rice grains for Zn (9.9–39.4 mg/kg) and Fe (9.6–44.0 mg/kg) fillings. Important genetic variations in aromatic and indica rice genotype for Fe and Zn were reported by Brar et al. (2011). Jalmagna and Madhukar rice varieties improved to deep and semideep water conditions with abundant in zinc and iron in grains. Similarly, rice grains of Basmati are also observed to carry a large concentration of zinc and iron. High Zn productivity rice genotypes such as BRRI dhan 62 and 64 have been identified through conventional breeding methods in Bangladesh (Harvest 2014). Wild species are used to develop nutrient-dense rice because they offer vast opportunity. No doubt conventional breeding approaches led a significant role in rice biofortification for Zn as well as for Fe contents, but conversely, due to some major constraints, most of the cultivars did not get attention from the farmers community.

- Few undesired characters including less yield or poor agriculture performance are observed by breeding of more zinc and iron varieties.
- Maximum iron improvement happens in the rice grain (aleurone layer) that is vanished through cleaning.
- For biofortified rice, the market does not provide different charges to farmers.
- Inadequate management enterprises to sponsor biofortified rice varieties.
- Lack of awareness among customers about the reputation of biofortified rice.

Some of these concerns are being addressed by current developments in genetics engineering and genomics, definitely the practical and logical interest. In the subsequent units, we have deliberated these methods which have been carried out to enhance micronutrients in rice.

4.1 Rice Breeding for Higher Iron and Zinc Contents in the Grain

Rice is cultivated in around 150 million hectares of the world, with 75% shared from irrigated areas. During overflow of water, numerous fluctuations take place in soil chemistry, such as decrease in iron concentration that formerly triggered as oxides and hydroxides, and convert into soluble stage (Fe^{2+}), therefore, also increasing its accessibility to plants (Ponnamperuma 1972). According to its genotype, Fe^{2+} absorption may be greater such that it becomes a source of toxicity in plants; consequentially, its productivity is reduced. Under flooded conditions, breeding methods are very significant for selection of rice genotype which has high ability to tolerate the Fe contents in soil.

A biofortification research revealed that rice seeds are produced by IRRI breeding program in the Philippines which have high iron contents in their seeds. Fe has already been screened for the bioavailability of transgenic rice ferritin in rats, with $FeSO_4$ diet being as efficient for hematocrit substitutions (Murray-Kolb et al. 2002). Ferritin is a natural source of Fe and it is highly bioavailable and causes the early

growth of plants and animals. Researchers have testified that transgenic rice traits have the ability to tolerate the surplus quantity of Fe in the soil. Researchers found that under flooded conditions, CK4 cultivar are best to tolerate surplus iron in soil. This is partly caused by reduced Fe accumulation in the leaves and by a higher photosynthesis of Fe in the presence of foliar tissue.

Under Zn deficiency, differences in rice cultivar were linked to susceptibility to HCO_3, particularly under elevated pH soil circumstances (Forno et al. 1975). The root development of inefficient rice cultivars may be inhibited by bicarbonate levels of 5–10 mM in the uptake of Zn, but this same situation may boost the root development in effective crop plants (Hajiboland et al. 2003). The greater Zn attainment by rice crop is linked with tolerance of soil HCO_3 (Hajiboland et al. 2003). The differential exposure for the zinc deficiency between soybean (*Glycine max*) and bean was linked to the limited translocation from the roots. The volume of translocation and efficiency of absorption of zinc around the roots were linked to genotypic differences. With a Zn deficiency, phytosiderophore releases in root exudates are increasing in grass species, possibly in order to adapt to zinc shortage. This physiological mechanism activates Zn utilization, and subsequently, the nutrients are more accumulated in the seeds.

Somayanda et al. (2013) carried out research on the effect of zinc on rice grain and tolerance against zinc deficiency. It is found that certain Zn effective rice variety has higher capacity to translocate the Zn from adult part of tissue to young developing part. This genotype is sensitive to the Zn deficit. In this manner, Zn-associated QTLs have been recorded, but neither of them has an impact greater than 30% in phenotypic variation (Stangoulis et al. 2007; Norton et al. 2014). Furthermore, QTLs associating grain zinc content were identified, and zinc uptake, transportation, and removal in the grains are better understood (Palmgren et al. 2008; Andersson et al. 2017). With reference to the restriction on iron accessibility in soil, two methods of Fe absorption have been developed. Strategy I, mechanism involves the removal of Fe^{3+} protons into the ground solution, Solubility of Fe^{3+} decrease by the Fe^{3+} membrane-bound chelate of reductase resulting in Fe^{2+} that transferred by Fe^{2+} transporters through plant root cells.

Numerous literatures have shown that the physiological and morphological reactions of crops fit in non-grasses, called Strategy I, fall under iron deficit situation. One of the reactions detected is the growth of root hair, via transfer and transition cell, which increases the interface between the roots and the center. The phenotypic change aids the release by the ATPases of more protons "triggered" by Fe transport responsive genes that acidify the central part to make Fe more soluble. Expression of Fe-reductase genes FRO and of Fe-transportation IRT genes is also present (García et al. 2011). Strategy II found in grasses is categorized by the development of Fe chelates; its major function is releasing the phytosiderophores and then following absorption of Fe^{3+} on a specific location of membrane used for their transportation (Marschner 2011).

Zuo and Zhang (2011) emphasized significant transgenic traits according to iron stuffing in edible part of the most cultured crops such as yams, manatee, beans, rice, corn, lentils, medicinal herbs, and wheat. Iron contents are important for humans,

and transgenic crops can enhance the cultivation of such crops. In general, cereal grains like wheat and rice have lower Fe levels than legume grains (Frossard et al. 2000). Depending on the variation, the capacity to increase the Fe content of rice grains is four to five times. Upon comparison between traditional and current rice, greater Fe is found in traditional grains, and less Fe is found in contemporary rice (Gregorio et al. 2000, 2008). The circumstance that shortest collection for the greater iron concentration in rice cereals did not belong in prior breeding database of rice can be recognized to this specific observation (Zuo and Zhang 2011).

4.2 Rice Breeding for Higher β-Carotene Content in the Grain

In the engineering of fortified plants, vitamin A plays an important role in genetic manipulation with a practical example of "golden rice." Surveys have been conducted to guarantee that β-carotene nutritive rice is used in developing nations for the deficiency of vitamin A (Beyer et al. 2002; Datta et al. 2006). Brown rice has enriched carotenoids and micronutrients, but its dietary value is significantly lost by the polishing technique (Tan et al. 2005). Controversial control of gene expressions of ferritin in rice was effective for rice grains as well as for refined grains in the increased concentrations of nutrient consumption (Vasconcelos et al. 2003; Khalekuzzaman et al. 2006). In the growth of golden rice, similar methods were operated (Datta et al. 2006).

At this time, ß-carotene content in polished grains is not present in any of the rice genotypes. Certainly, rice having genetic variation of the carotenoids could be used (Tan et al. 2005). In southern nations and Southeast Asia, more than half of all females and children are anemic. It reduces the mental development and growth in infants, and rises the rate of death during birth in anemic females severely. Though, bioavailability of β-carotene should be thoroughly investigated. New trials show a timing of iron's endogenous uptake from cereals to two times with a tiny complement of vitamin A or β-carotene (Graham and Rosser 2000). Rice is rich in β-carotene, and it may therefore decline vitamin A inadequacies and prevent anemia from shortage of Fe (Fig. 1).

4.3 Rice Breeding for Higher Folate Contents in the Grain

Vitamin B complex shortage is interlinked with folate deficiency leading to many health issues. Enforcement of industrial food supplement and the use of folic acid pills are options; however, in developing states, it may not be viable. Recent developments indicate that biofortification of farm foodstuffs to increase the content of folate is a feasible approach to fight against shortage of folic acid worldwide. Biosynthesis of folate is assumed adequately by the enzyme and genes. Metabolism and the outcomes of preliminary research in plant genetic engineering are thus encouraging (Bekaert et al. 2008). These may decrease vitamin A deficiencies and defend against anemia.

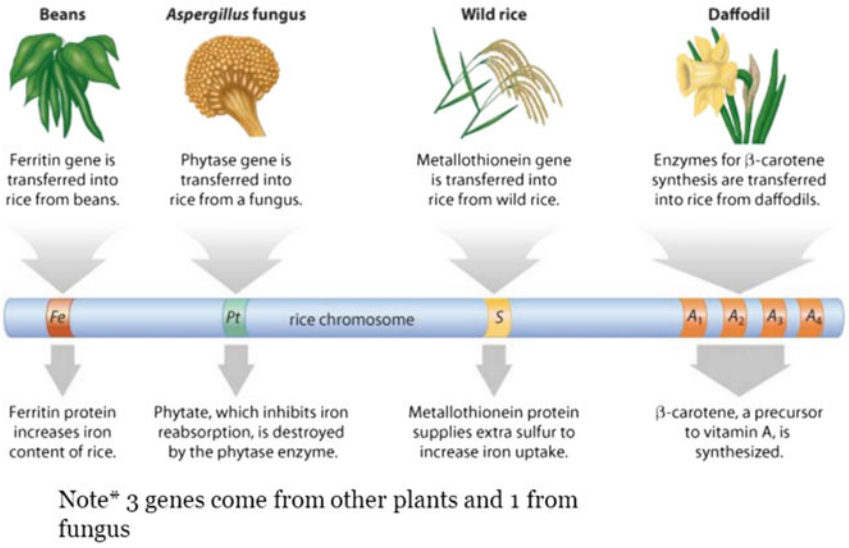

Fig. 1 A schematic way of the formulation of golden rice

Formation of folate is identical in microorganisms and crop, and in both groups, the genes which are used in enzyme pathways are all identified (Storozhenko et al. 2007a, b). Similarly, some part of glutamate, pteridine, and p-aminobenzoate (P-ABA) basically are three segments of the tetrahydrofolate (THF) molecule. THF process takes place in cytosol of bacteria, but in crops, three different organelles are involved such as mitochondria, cytosol, and plastids (Jabrin et al. 2003). The crops are able to cope with folic acid instability, and their degradation rate can be reduced.

In this phase, the preliminary reports on the expression of cyclohydrolase I enzyme identified as GTPCH I are capable for tomato fruits and *Arabidopsis thaliana* (De la Garza et al. 2004). In both situations, the pteridine levels in transgenic *Arabidopsis* and *Solanum lycopersicum* (tomato) were 140 times advanced as compared to wild type with gene bank genes BE136861 and AE000304, respectively, but the increment in folate quantity was only two to four times, which showed that this metabolism had to be further engineered. The result was an increase in pteridines. Transgenic tomato crops were subject of a decrease in p-ABA, which led to a rise in folate content with an extra 2.5–10 times with an exogenous shipment of GTPCHI overexpression. This remark shows not only the requirement for pterine and p-ABA (folate precursors) to raise their concentration in folate simultaneously but also the high physiological potential in plant cells (De la Gazra et al. 2004).

5 Genomic of Micronutrient Biofortification

The foremost yield plant is rice which have full sequence of genome. Since 2005, the rice preliminary genome has been made available in International Rice Genome Sequencing Project. In genomic rice, huge developments have been completed. Recently 3000 rice genes have been re-sequenced from 89 countries, which recommended enormous variability amid the rice genes. This information can be used for extensive finding of innovative alleles for improved cereals biofortification. Through several scholars, several genes and QTLs have been found which are tremendously beneficial for refining vitamin A, Amylose content, fragrance, protein, β-carotene, folate content, Fe and Zn content of rice cereals in current genotypes (Table 1). Techniques such as epigenetic and small RNA noncoding are used for developing the biofortification of the grains because these are the best option for researchers to explore their genetic variability.

5.1 Quantitative Trait Loci for High Fe and Zn

Classification and identification of chromosomal fragments (frequently indicated as quantitative trait loci (QTL)) that support in rice grain to accumulate zinc and iron that can cover the methods for MAS (marker-assisted selection). Controlled by a huge number of genes, QTLs are genomic loci regulatory quantitative characteristics with slighter involvement to the traits developing inconstant, as opposed to discrete, variety (Liu 2017). Reliable strategies to break down iron and zin in rice endosperm samples are critical to QTL mapping. This has facilitated to identify a few genes related to Fe homeostasis in rice. Hereditary concerns can be addressed by the encounter of genes/QTLs associated with transport, absorption, and stacking of zinc and iron in rice endosperm and identification of their corresponding expression relying upon the lack or adequacy of the components in the cell and root atmosphere. This is accepted to be valuable in creating systems for increasing micronutrient contents in grain (Gande et al. 2014).

With the help of varying genetic backgrounds aided by intraspecific and interspecific crosses, in rice, QTL mapping trainings for high grain zinc and iron have been achieved (Anuradha et al. 2012; Swamy et al. 2016). In RIL population, 14 QTLs controlling zinc and iron contents were observed, and these were developed from Madhukar × Swarna (Anuradha et al. 2012). In rice, QTLs influence both Fe and Zn quantities in grains co-localized on chromosomes 7 and 12. Similarly, in unpolished rice Swarna × Jalmagna, RILs have been utilized to record QTLs for Fe and Zn quantity, resulting in the association of the markers RM3322 and RM7488 for Zn and Fe (Kiranmayi et al. 2014). In maize mapping population, QTLs co-localization disturbing grain zinc and iron quantity has been identified (Qin et al. 2012). In cereal grains, these suggest that contents of zinc and iron can be improved instantaneously by focusing on the identical chromosomal zones. In order to identify applicant genes for grain zinc and iron, meta-analysis of QTLs was done for rice and maize. In rice, 22 MQTL while in maize 4 synthetic MQTL associated sections and 3 MQTL

Table 1 Traits related to rice biofortification with their corresponding genes

Traits	Gene/breeding approaches	References
Vitamin A	Maize genes *crt1 and psy* from *Erwinia uredovora*	Paine et al. (2005)
	Daffodil genes *crt1*and *psy* from *Erwinia uredovora*	Ye et al. (2000)
Amylose content	AP2/EREBP	Fu and Xue (2010)
	SBEI and SBEIIb	Yongwei Sun et al. (2017)
	Waxy	Chen et al. (2010)
	OsGBSS1	Liu et al. (2014)
	FLO2	Wu et al. (2015)
	OsAGPase	Lu and Park (2012a)
	OsBEIIb	Lu and Park (2012b)
Iron	*OsNAS2*	Lee et al. (2012)
	OsVIT2	Bashir et al. (2013)
	OsYSL9	Senoura et al. (2017)
Zinc	OsZIP4	Ishimaru et al. (2005)
Iron and zinc	*HvNAS1*	Masuda et al. (2009)
	OsIRT1	Lee and An (2009)
	OsNAS	Johnson et al. (2011)
	HvNAS1	Masuda et al. (2009)
	OsZIP8	Lee et al. (2010)
	Mx*IRT1*	Ishimaru et al. (2005)
	AtIRT1	Boonyaves et al. (2016)
Fragrance	BADH2	Shao et al. (2013)
Protein	Two new genes are artificially synthesized by fusing endogenous rice genes with lysine/threonine motif (TKTKK) coding sequences	Jiang et al. (2016)
β-Carotene	*ZEBRA2*	Chai et al. (2011)
Folate content	*Arabidopsis* GTP-cyclohydrolase I (GTPCHI) and aminodeoxychorismate synthase (ADCS)	Storozhenko et al. (2007a, b)

holding applicant genes were exposed by the literature. The maize orthologs of rice, GRMZM2G366919 and GRMZM2G178190, were marked as NRAMP (natural resistance-associated macrophage protein) genes. For biofortification, these genes were recommended as applicants upon phylogenetic analysis, accountable for the natural variant of zinc and iron quantity (Jin et al. 2015).

Inside the QTL section, efficient validation and expression analysis via knockout or overexpression of applicant genes can be utilized to illustrate sequence of gene. Due to accessibility of genomic sequence of rice, it has potential to detect the genes causing QTLs. Anuradha et al. (2012) detected ten candidate genes underlying iron and zinc QTLs, included in more than one steps of transport, collection and absorption of iron in zinc in seeds. In high and low zinc and iron lines, the expression pattern of these candidate genes was observed by Agarwal et al. (2014). They showed significant correlation of seed Fe and Zn concentration with the expression of genes causing QTLs. Eight candidate genes were detected by Chandel et al. (2011) which were expected to be included in homeostasis of zinc and iron, previously, the five QTLs recorded in RIL population produced by Lu et al. (2008). According to data on genes, functional markers can be produced for acceptable QTL mapping and MAS (marker-assisted selection) breeding methods for grain nutritive character.

5.2 microRNAs

Besides the coding genes of protein and noncoding small RNAs called as microRNAs (miRNAs) are the potential source for increasing zinc and iron concentration of grains. Nearly these miRNAs are utilized in all metabolic and biological processes in plants, for example, in plant reaction against stress, plant growth, and biosynthesis of the cell wall (Mangrauthia et al. 2017). They are subdivisions of approximately 22 endogenous nucleotides, minor, noncoding, controlling RNA fragments that control the expression of gene by facilitating degradation of messenger RNA and translational suppression in a sequence particular method. In plants, numerous miRNAs have been described to be included in the transport and absorption of micronutrients (Fischer et al. 2013).

Plant adjustment to phosphate and sulfate deficit is regulated by several miRNAs according to recent studies. However, there is a limited research on the function of miRNAs in adjustment of zinc and iron in rice seeds. Currently, miRNA expression analysis associated with biogenesis of microRNA and homeostasis nutrients displayed the function of these small RNAs in the adjustment of zinc and iron in rice metabolism. As the result of miRNAs downregulation in the upregulation of their assumed focus genes showed the contribution of miRNA facilitated adjustment of iron homeostasis. Downregulated miRNAs important for iron deficit such as miR398, miR395 and miR169 were also described as responsive to deficit of other nutrients such as Cu, S and N which furthermore recommended that these miRNAs might be associated with pathways of signal transduction. miR172, miR169, miR171 and miR156, were known as miRNAs responsive iron deficit (Agarwal

et al. 2015). For example, under iron deficit, miR158 increased more than fourfold in the phloem in *Arabidopsis* (Buhtz et al. 2010). Recently, iron deficiency-responsive miRNAs was found in *Arabidopsis*. Of these, some were seen to downregulate while others upregulated in the stage of iron deficit both in roots and shoots (Kong and Yang 2010).

In *Arabidopsis*, storage of protein- and iron-associated transporters was explained by miRNA-mediated regulation. In copper deficit, one of the important precursors for Zn/Cu superoxide dismutase is miR398; these were controlled via iron deficit but in contradictory method. Fe deficit decreases the expression of miR2111, miR408, miR399, miR398s, miR398c, miR398b, and miR398a, while copper deficit enhances their expression and, sequentially, controls the expression of CSD1 and CSD2 (Waters and Troupe 2012). Consequently, the iron-copper interassociation is additional unique discovery concerning the learning of gene expression in homeostasis of iron.

In *Arabidopsis* from the library of small RNA population, eight miRNAs from five families were formerly known as iron-responsive families. Interestingly, the iron deficit responsive cis acting element 1 and 2 (IDE1/IDE2) is similar to the iron-responsive gene families which controlled the iron deficiencies (Kong and Yang 2010). In *Sorghum bicolor*, deficit of zinc was detected to enhance the upregulation of numerous miRNA families, for example, miR319,miR166, miR171, miR172, miR399, and miR398, in order targeting several gene family members with transporters (Li et al. 2013). Adaptive mechanisms of plants include the downregulation and upregulation of various miRNAs in metallic stresses, for instance, Cd, Hg, and Al stress (Zhang et al. 2013).

Molecular pathways related by metabolism of iron involving other genetic pathways can be decoded by the interaction of miRNAs and regulated targets. There is a need to focus on the function of miRNAs in rice homeostasis of iron and to utilize these hereditary elements for increasing zinc and iron concentration in seeds. An effective alternate strategy includes engineering of miRNA expression levels. Extensive studies should focus on the function of miRNAs in controlling particular transcription factors and transporters in iron diet. miRNAs carry significance for character enhancement in yields, a matter that has been currently studied by Tang and Chu (2017).

5.3 Epigenetics

Various mechanisms associated with epigenetic regulation control the chromatin structure which regulates the material about the genomic expression in the cell. It includes variants of histones and their posttranslational modifications and methylation of DNA. Epigenetic modifications within the DNA sequence can be induced by stress signals, which in turn control the gene expression related to pathways of stress (Chinnusamy and Zhu 2009). The upregulation of several LTR retrotransposons is brought about by iron stress. LTR retrotransposons are shown to take part in the transcriptional reaction to stress and could therefore discuss an adaptive benefit for

the plant (Finatto et al. 2015). A current report by Duan et al. (2015) studied the function of iron in demethylation of DNA. According to them, the ROS-1 repressor of silencing mediated active demethylation of DNA needs MET18-dependent transfer of the S-Fe cluster increasing the significant function of CIA path in epigenetic regulation. More insight into the function of epigenetic regulators in homeostasis of zinc and iron in rice would pave ways for biofortification in the future. Bisulfite sequence of genome and immune precipitation of chromatin are some genomic advances that have facilitated in resolving the source of epigenetic regulation of genes related to development and stress.

6 Genome Engineering

Engineering of genome for enhancing Fe and Zn content in rice grain is the ultimate utilization of information obtained from genomics studies. Genomic adjustment and engineering could be either targeted or random. The methods for random engineering of genome comprise of molecular breeding and mutagenesis, while targeted engineering of genome contains editing of genome and transgenic. In this segment, we confer the methods of genome engineering which have previously been proved for increasing zinc and iron. Similarly, the opportunity of utilizing developing biotechnological methods, for instance, editing of genome for biofortification, is briefly described. In latest years, genetic plant transformation is one of the biggest developments in agricultural technology. Development of genetically modified (GM) crops has a major financial significance because new agronomic features are incorporated in the crops, such as food quality enhancement, resistance against herbs, phytopathogens, or resistance against insets (Pereira and Vieira 2006). Different methods are used for the transformation of plants such as in vitro selection. This process works by incorporating DNA into plant cells via microorganism such as bacteria and viruses. It is a biological method or a direct physical method. There are three common methods which are used for plant transformation such as *Agrobacterium tumefaciens*, bioballistics, and protoplasts. In various plant species, genetic transformation has now already implemented various features of socioeconomic concern. These features are primarily directed at enhancing plant efficiency against biotic and abiotic stresses. In transgenic plants, characteristics associated with plant development and product quality can also be changed. This is a trend to improve the number of features to be manipulated by genetic engineering and to boost the variety of products for farmers and consumers.

6.1 Genome Editing

A powerful tool to finding out gene function is targeted gene mutagenesis which also facilitates hereditary development in rice. Editing of genome includes forms of engineering that are nuclease-based, for instance, CRISPER/CAS or TALENS, to produce mutations, accurate incisions, and substitutions in eukaryotes particularly in

plants. Various reviews are currently issued to focus on the basics of genome editing methods and their uses in plant yields such as the modification of target genes (Noman et al. 2016; Liu 2017). TALENs are comprised of engineered specific DNA-binding domain of a TALE merged to a general FokI cleavage domain. Its initial application was completed by Li et al. (2013) for trait development in rice. A mutation was presented in the promoter area of the sucrose efflux transporter gene, *OsSWEET14*, leading to increase resistance to bacterial disease. The TALEN scaffold was optimized by Zhang et al. (2013), and further accomplished TALEN-mediated gene was substituted in the ALS gene of tobacco protoplasts. Within barley phytase gene HvPAPhy, an area that covers a cluster of regulatory motifs in the promoter was focused on by Wendt et al. (2013). They revealed that TALEN-induced double-strand breaks resulted in the induction of short removals on the target place; therefore, a range of various mutations were brought about in each barley transformant, which showed that mutations happened self-sufficiently in diverse cells. In *Arabidopsis*, according to Christian et al. (2013), mutations induced by TALEN were transferred to the next progeny at ranges 1.5–12%. Fragrant rice containing 2AP (2-acetyl-1-pyrroline), a main fragrance compound, was developed using TALENs targeted upon *OsBADH2* (Shan et al. 2015).

Based on its higher effectiveness and facility, editing of genome by CRISPR/Cas has gathered well-known awareness nowadays. A detailed information of protein engineering is required in TALENS and other methods about editing of genome to produce specific ruptures in DNA. However, CRISPR/Cas is built on identification of focus DNA by short oligos identified as sgRNAs. With the help of targeted mutagenesis of CRISPR/Cas9 of the ERF transcription factor gene *OsERF922*, improved resistance blast in rice has been accomplished. As a result, at both tillering and seedling phases, after pathogens attack the quantity of lesions, blast was made lower in mutant-type compared to wild-type plants (Wang et al. 2016). Homozygous rice plants that have resistance to herbicides via the CRISPR technique were recently developed by Sun et al. (2016). The feasibility of the application of CRISPR in accurate gene substitute is demonstrated, which is needed for biofortification of rice. Similarly, zinc and iron genes accompanied by their promoter sequences could be modified which are comprised in transportation, absorption, and storage of the nutrients. Several candidate genes involved in zinc and iron homeostasis were recognized by Anuradha et al. (2012) and Agarwal et al. (2014). These carry significant functions in Strategy I and II obtaining ferric iron from soil and long-distance transportation in leaves and storing iron in endosperm. There exists a great advantage in gene editing related to zinc and iron concentration for biofortification of rice seeds done by the current tools and methods due to their specificity and high accuracy. Creation of a desired allele and acceptable alteration in the expression of focus genes according to the requirements through modifying regulatory elements of genes can be done using genome editing. This technique is beneficial in increasing the grain zinc and iron concentration. There is possibility that small nucleotide modifications through genome editing present in new varieties of crops will be exposed to the identical biosafety parameters that are occupied for current GMO crops. However, with respect to trait improvement, this technique is still in its

infancy; it has been successfully demonstrated for improvement of few traits in crops, for instance, maize, rice, tobacco, and barley.

6.2 Transformation via Protoplasts

Protoplasts can be regarded as perfect for transformation. Due to the lack of the cell wall, DNA sequences can infiltrate into protoplasts freely, such as regeneration and competent cells (Lin et al. 2018). This transformation technique can be performed chemically with polyvinyl alcohol or polyethylene glycol and electroporation that involve pore formation within the membrane by quick electric pulses of elevated voltage. Somehow, this technique is not acceptable in regeneration of plants due to its limitations because a few diverse plants have high rate of regeneration (Fig. 2). A significant achievement of protoplasmic transformation was seen in rice, in which genes of both the indica and Japanese subspecies were acquired from transgenic plants with phosphotransferase hygromycin and glucuronidases (GUS) (Zhang and Wu 1988).

6.3 Transformation via Bioballistics

In most plant species particularly angiosperms and monocotyledons, infection is carried out by *Agrobacterium*. However, this bacterium has some restrictions because its vector is used for transformation of genetic material. Therefore, direct transformation (chemically or physically) was established corresponding to *Agrobacterium* transformation method.

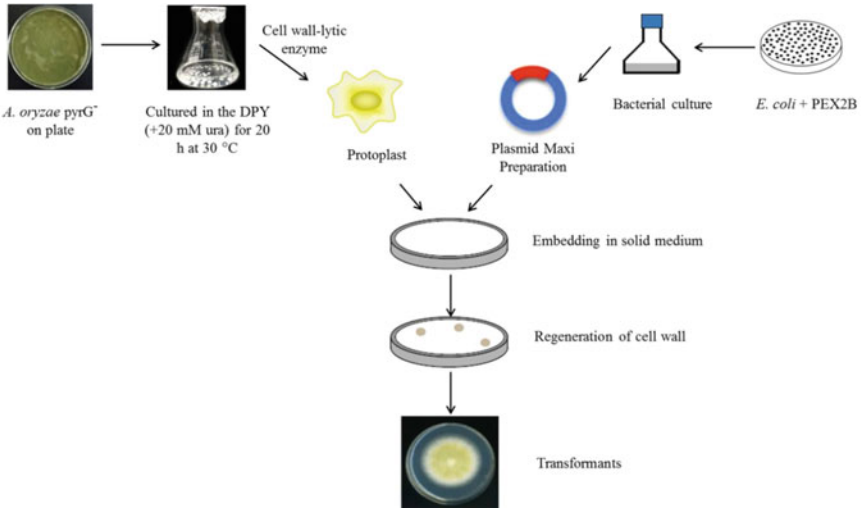

Fig. 2 Transformation of plant through protoplast

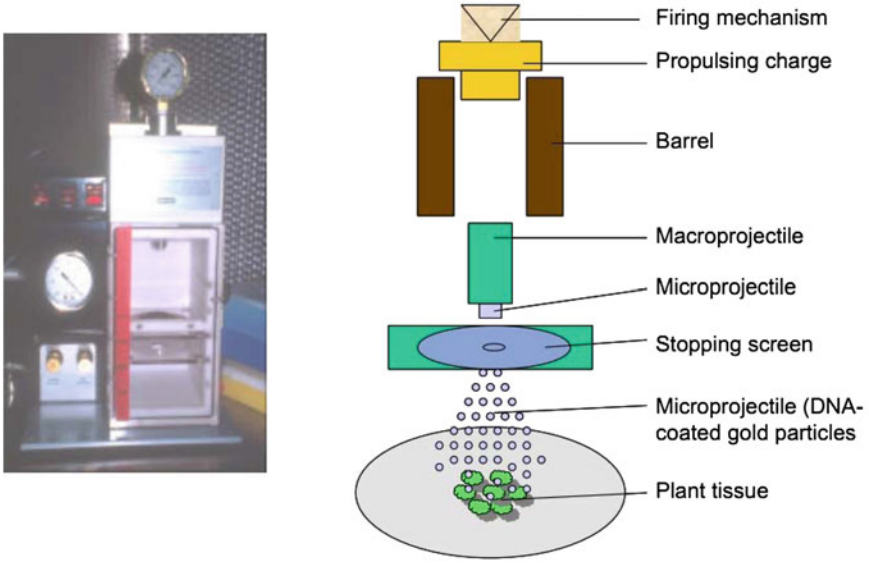

Fig. 3 Transformation of plant through biolistic method

One of the major methods for the transformation of plants was the bioballistics method or the bombardment with particles with high possibility of microorganism and animal tissue being transformed (Klein et al. 1992). This method is used for many plants because it provided target tissue in in vitro regeneration. Many particle acceleration devices were proposed. Nowadays, particle acceleration using membranes of a varying thickness is the most common model with electrical release or air pressure (Fig. 3). Bioballistics does not confide in genotype and its contact with bacteria in contrast to the *Agrobacterium* transformations, but relatively, it is very helpful to transfer genetic material in in vitro regeneration system (Pereira and Vieira 2006).

6.4 Transformation via *A. tumefaciens*

The most commonly used transformation approaches in GMO plant development are mediated by bioballistics and *A. tumefaciens*. *Agrobacterium* is a soil bacterium which is capable of transferring fragments of DNA in plants and has become a source for transferring of plants. T DNA in plant and measurements of these altered cells to differentiate into a plant involve obtaining the transgenic plant (Fig. 4). The regeneration plants are developed by totipotency, ability to differentiate, via in vitro tissue culturing method. The progress in molecular biology is essential both for the clarification of the depths of the molecular basis of agrobacterium-host interaction and for the creation of tumor-inducing plasmid transformation vectors. Transgenic

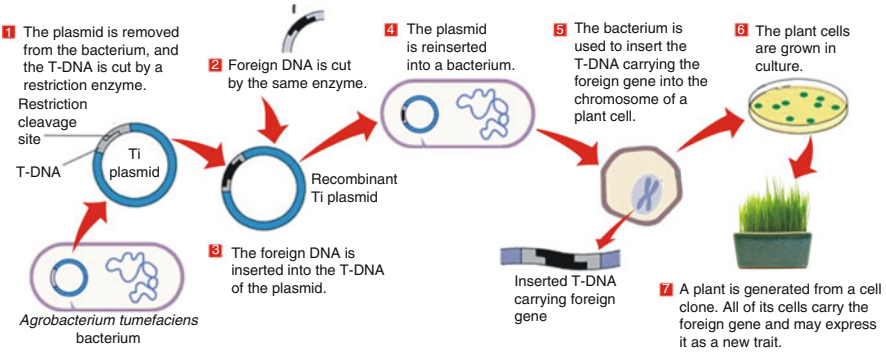

Fig. 4 Transformation through *A. tumefaciens*

plants are attained by the combination of basic technique of molecular biology and in vitro tissue culturing.

6.5 Identification and In Vitro Selection of Transformed Tissues

Scientific or agronomic interest of genes as well as marker or reporter genes is used for the identification of transformed plants. Reporter genes confirm that the process of transformation is properly carried out through the identification of cells and tissues transformed. These genes enable for a temporary expression of gene, when these are not merged into genome of cell. Marker genes are used to identify or differentiate between transformed and non-transformed cell. Such genes are implemented to promote their identification by being in slight contact with the genome of cell under transformation. Mostly antibiotic-resistant genes are used which help to screen transformed cells that ultimately regenerate a plant.

Generally, reporter gene that is used is *UidA*, encoding the enzyme of β-glucuronidase (GUS) that gives a fluorescent or histochemical expression for the transformation of genetic material. Furthermore, generally utilized reporter genes are green fluorescent protein (gfp) which is isolated from the *Aequorea victoria* commonly called jellyfish and luciferase genes (*luc*) which are extracted from *Photinus pyralis* commonly called eastern firefly. The most commonly used genes in the detection of transformed tissue are luc and gfp. Development of sensitive and accurate detection methods such as imaging apparatus and photodetection has helped in luciferase genes for in vivo visualization and classification of tissues and cells in real time; to study circulation of pathogenic microbes in animals and plants; as indicators of tumor cells, in the development of new therapies; and as environmental biosensors to detect heavy metals and insecticides in polluted waters (Viviani 2007). luc and gfp is one of the latest genes which are used for the study of protein, because these reporter genes are involved to permit its gene position (root, leaf or

fruit) of existence for the expression that linked to regulation of gene. The expression methods enable the evaluation of the presence or lack of certain nutrients in some circumstances, which are capable of the selection of genotypes; hence, ultimately, gene expression can be evaluated.

6.6 Concepts of Gene Expression and Regulation

In many areas of biological research, the study of gene expression is very significant. Information about patterns of gene expression should improve the understanding about complex regulatory systems and may lead to the recognition of genes applicable for plant breeding. PCR brought many developments and scientific benefits for the gene expression and sequence of genome. Amplification of specific fragment of DNA is carried out by this method using the specific complementary primer of the target DNA sequence.

The RT-PCR method is used to directly amplify the coding region of mRNA molecule. After isolation of RNA, the reverse transcriptase enzyme (viral origin) is used to synthesize mRNA from cDNA. RNA membrane from the mixture RNA/DNA is digested by RNase, so DNA polymerase is used to replicate cDNA. Both RNase protection analysis and Northern blot analysis are used for the quantification of mRNA, but RT-PCR can permit for the quantification of mRNA in very minute samples. However, this method is very significant for the single-cell RNA quantification (Weis et al. 1992). In research and clinical labs, RT-PCR is used compared to conventional PCR because RT-PCR results are rapid and precise, but the innovation of this technology is obtained from the conventional PCR. Several approaches for the detection of differential genes on a large scale have been created. The most widely used technique is microarray; it depends on the quantification of RNA by gene expression products, and because of this, it offers a strong platform for evaluating thousands of gene expression pattern simultaneously.

Relatively, mRNA is isolated from the tissue, and it is used in microarray technique. The messenger RNAs have been converted into complementary DNA. cDNA pools are labelled with the group of fluorescents. In microarray technique, samples are denatured by heat after labelling. For studying it, the cDNA pools are classified into groups and put into the microarrays by immersing a chip into hybridization solution. Both the tissue of cDNAs considered and the "pores" of slide are in the form of a plane ribbon. If sequences are complementary, the marked cDNA strands hybridize to a "pore" of the cDNA of the slide. Microarray chip is put into dark room and exposed with laser after drying and washing off. The radiations will be captivated by fluorescent markers. All markers release radiation at a different wavelength, so that any "pore" of the slide can quantify hybridized cDNA. As gene fixed in each of the "pores" is known, it is possible to know the expression of a control tissue in relation to the variant. Computationally, colors of each marker are

allocated according to their range of emission, and it facilitates in determining the level of gene expression (Hajiboland et al. 2003).

Parallel analysis of miles of genes in two populations of labeled RNAs is possible by the microarray technique, whereas at the same time RT-PCR measures the gene expression of limited genes from different samples; that way, this technique is used for few cell expressions. When these modern techniques are compared with the conventional techniques (in situ hybridization, ribonuclease protection assays, and Northern blot), the numerous advantages of high rate of automation and its speed are observed. In situ hybridization is a technique used for identification and finding of specific sequence of nucleic acid of the prepared and preserved cells or tissue with the help of probe of sequence of interest. This technique permits a localization of the copies of the cell inside a tissue, although it is a complicated method. According to the study on cell and tissue, in situ hybridization is based on the same principles of DNA/RNA in which probe is used to distinguish specific sequence of nucleotide (Parker and Barnes 1999).

6.7 Protein Expression

One of the primary goals of diagnostic and science procedures is the identification of particular proteins. Sequence of nucleic acids and unique proteins is identified by blotting methods. Three primary blotting methods were created frequently that are referred to as Southern, Northern, and Western blotting which allow the analysis of DNA, RNA, and protein, respectively.

Southern blotting enables for the identification of DNA fragments with DNA probes that are hybridized through H-bonding to make opposite chromosomal DNA pieces. Restriction endonuclease enzymes are used to generate the DNA fragments by chromosomes. These enzymes are extracted from microbes, and dsDNA are digested by these enzymes (Southern 1975). The steps of northern blotting are similar to the southern blotting instead of DNA probe in which RNA probe is used. Northern blotting (Thomas 1983) enables the identification and measurement of mRNA by the hybridization of their equivalent DNA sequences. Gel electrophoresis is used to separate the mRNA on the basis of size and then is transferred on filter paper; formerly, the probe of genes is used to find out the interest of RNA. Western blotting is used to identify a protein by specific antibodies. Under analysis, specific antibodies are used to locate the protein in Western blotting.

6.8 Proteomics

Although it seems relatively difficult to identify all encoded proteins from the genome even from the simple organisms, the scope of proteomics is increasing day by day (Suresh et al. 2005). The study of proteomics is linked to different methods such as regulation of syntheses of protein, cell signaling pathways, and posttranslational modification, as well as the critical steps of pathophysiological or

physiological condition in organisms of the cell. At the start, the entire amount of protein was considered under the study of genome, but Wilkins et al. (1996) proposed the term proteome. The scientific community realized that after the rapture produced by sequence of genome of various organisms, significant studies of the protein expressed are essential in order for gene functions to be fully understood. Two-dimensional electrophoresis and mass spectrometry are used in proteomics.

Electrophoresis worked on the principles of electric field, it is used for the separation of the molecules on the base of charge. In 1937, Arne Tiselius first used this technique for the analysis of the protein, who invented a technique known as free electrophoresis, which involved the breakdown of blood serum into five primary protein segments for which he was awarded Nobel prize. This method has been constantly improved in recent decades, allowing more accurate analyses, such as denaturation of 3-D polyacrylamide gel and 2-D electrophoresis.

The common method which is used to examine the molecular weight of oligomeric protein is polyacrylamide gel electrophoresis, and it works in the existence of SDS (sodium dodecyl sulfate). The medium of gel is consisted of cross-linking of polymer of N acrylamide; it is spongy in nature so selected material or molecules pass through it. The network of pores depends on the quantity of acrylamide; smaller pores are formed by a greater quantity of acrylamide and vice versa. After processing with amphipathic detergent, on the top of the gel, proteins are implemented, and then the gel is exposed to electric current which allows them to migrate in the direction of positive end via the acrylamide mesh. According to size, each protein will shift in a different way, for example, smaller protein will migrate quicker, although bigger proteins will have more trouble in moving through the gel mesh and consequently this process slows down. In case of electrophoretic mobility, a plot is drawn in contrast to logarithm of molecular masses of identified polypeptide protein, and a straight line is attained which is considered as standard curve. This is then used to find out the molecular masses of subunits of desired proteins.

6.9 Mutant Study

Evolutionary processes and plant breeding are based on genetic variations which are significant for the effectivity of the artificial and natural choice (Jennings 1985). The germplasm that contains preexisting genetic variation can further build on changeability via recombination of gene, soma-clonal and artificial mutations, and genetic transformation. Above five decades, various strategies are used to produce many rice cultivars from induced mutations and choices of genetic components from transformed populations. Usually, point mutation may recover qualities permitting the choice of hereditary traits carrying superior compositions in the initial productions. This method changed the frequency of some negligible genes with the expression of characters in the IAC-24 wheat genotype which took into consideration the determination of at any rate eight lines that kept up the first attributes and had characteristics, such as the resilience to pathogens and tallness of plants (Neto

et al. 1995). Induction of mutations efficiently reduced the plant height especially which generated via synthetic crosses in *triticale* (Pandini et al. 1997).

In Brazil, ^{60}CO is used to produce hereditary mutation in the Taim "BRS 7" rice genotype. This rice cultivar characterized by erect and short leave and atypical tallness nearly 80 cm with high tillering limits belongs to the modern group (Filipino). 623 mutants were chosen for varying characters from M2 generation, including both the cycle and plant height (ZIMMER et al. 2003). In rice mutants, Martins et al. (2005) studied the changeability of morphological characters. They observed that exposing seeds of the cultivar BRS 7 "Taim" with ^{60}CO to radiations generated mutants for characters such as cycle, no. of tiller, plant height, index of fertile tiller and number of panicles.

6.10 Patch Clamp

Alongside genetic breeding, various techniques focusing on proteins located on the surface of membrane and its role to facilitate the nutrients may be utilized for producing plants which have high nutritional rate. One such technique is the patch clamp method consisting of recording chains that flow through a small part of plasma membrane (Barry and Lynch 1991). A suitable electrolytic solution of 4–6 MΩ resistance in a glass micropipette when pushed alongside the membrane is liquefied by it. This forms a seal with extremely mechanical stability and high resistance. Finally, the pressure ruptures the membrane cover, while the pipette is still closed to the cell supplying entrance to its central part. A high potency seal is needed because, firstly, the higher the power, the more comprehensive is membrane cover filling relating to the exterior solution and, secondly, a high strength decreases the chain that can fix between the layer and pipette. Therefore, all the particles must pass in pipette when the ion passage opens. An ultra-sensitive enhancer coupled to the pipette can then be used to measure the resultant electric chain.

According to Fuchs et al. (2005), OsAKT1 (rice K^+ uptake channel) is delicate to salt stress. Patch clamp technique is utilized to expand root protoplasts of rice. This method was used to identify a K^+ incoming rectifier having comparable properties of channel during the OsAKT1communicated heterologous. This technique was established to analys the performance of ion channels and its subsequent use in plant breeding that helps in the choice of persons by a higher number of specialized transporters for particular nutrients in root tissues. Similarly, patch clamp method can additionally support to discover the probability of membrane transporters to be merged in developed species, therefore finally recovering the absorption effectiveness of nutrients even when soils lack them (Table 1).

7 Final Considerations

Micronutrients biofortification of rice is high priority for researchers, industries, and policy makers of countries where rice is a primary source of calories. In Asia and Africa, rice with micronutrients can be a major source to decrease malnutrition. In addition to being a main essential food, rice has the benefit of being a model yield which has been considered broadly in contrast with other crops. As a result of the initiation of genetic engineering, currently, it is significantly possible to produce a rice crop with high quantity of β-carotene in its seeds. This product was successfully obtained due to the vast information of the enzymes linked with biosynthesis of β-carotene. Therefore, rice breeding coupled with genetic engineering and biotechnology is helpful in biofortification of the rice grain. Breeding programs should make use of plant nutrition concepts alongside the knowledge on genetics, particularly the biomolecular characteristics of plant nutrition. In developed rice, biofortification of Fe and Zn is improved via genetic traits and accessibility of landraces and wild relatives, hence taking natural allelic varieties. Different methods, for instance, mapping QTLs and genes for high zinc and iron, have been facilitated to find out vital genes that can be focused on for enhancing zinc and iron concentrations in rice via transgenic and traditional approaches. On the other hand, MAS has not been utilized until now to improve zinc and iron quantity; still numerous QTLs have been recorded. With the purpose of explaination, the system of genes desired to boost nutrient accessibility in granules, Omics approaches may also be used. For the absorption of zinc and iron, various genes have been involved. Numerous genes have been considered for their function in translocation, absorption of zinc and iron, storage and filling in endosperm. Approaches such as transcriptomics, DNA sequencing, SNP, RNAi discovery synchronized with exact genome engineering tools can be utilized for attaining the goals of modified zinc and iron in seeds rice.

Additionally, in rice genotype, plant miRNA metabolism should be explored especially in zinc and iron stress. In rice, miRNAs facilitated the collection of zinc and iron. CRISPER/CAS9 genome altering tools can be used for future crop improvement. Editing of genome can be utilized for launching and producing new alleles or helping knockout of negative mechanisms to increase zinc and iron concentrations in rice. In plant stress reaction, the role of DNA methylation and epigenomics may be utilized to develop dissimilarities for biofortification.

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Improvement of Nutritional Quality of Rice Seed Through Classical Breeding and Advance Genetic Engineering

Subhankar Mondal, Dipak Gayen, and Subhasis Karmakar

Abstract

Rice (*Oryza sativa* L.) is a staple food crop for a large number of human populations whose diet solely depends on rice. Rice contains sufficient amount of starch but deficient with several kinds of nutrients like iron and zinc and contain least amount of protein as well as essential amino acids like lysine, tryptophan and methionine and lack beta-carotene. To improve the nutritional content of rice, several strategies have been implemented through biotechnological techniques and conventional breeding program, which include the overexpression of iron storage protein *ferritin* or by heat-stable *phytase* gene, overexpression of *NAS* gene family members like *NAS1* and *NAS2*, incorporating beta-carotene pathway inside the rice, overexpression of some proteins with high lysine content along with some genes from other organisms, incorporation of some synthetic proteins or by manipulating the storage of prolamins and glutamines proteins and crossing with the high nutrient or protein content rice varieties with high yielding ones to produce high yield nutrient, protein varieties. Besides, several approaches have been adopted to develop a low glycaemic index (GI) rice to mitigate type II diabetes. The content of ω-3 fatty acid has been increased in rice to suppress cardiovascular and autoimmune diseases. Apart from biofortification, genetically modified rice has been developed by silencing of the desired gene using RNAi technology to increase the shelf-life. During the

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last decade, several genome editing approaches, including CRISPR-Cas9, contributed a significant role in biofortification process of rice as well as other crops.

Keywords

Lysine content · *OsAAP6* · *OsWAXY* · 13KDa prolamin · *RINO1* · *LCT1*

1 Introduction

Rice (*Oryza sativa* L.) is an essential food for a large number of human populations all over the world. However, rice contains sufficient amount of carbohydrate but deficient with several kinds of essential nutrients, amino acids like lysine, tryptophan, methionine and inadequate source of vitamin A. Deficiency is related with several types of abnormalities like anaemia, which is related to iron deficiency. Zinc deficiency is linked to growth retardation, abnormal immune function, etc. Vitamin A deficiency is characterized by night blindness and low protein, imbalanced diet caused ageing. Therefore, nutritional improvement of rice plays a vital role to fight against several human disorders as well as malnutrition, which is the main reason behind all kinds of diseases. To improve the nutritional content in rice seed, several works had been performed through modern biotechnological techniques and conventional breeding programme which includes the overexpression of iron storage protein ferritin in tissue-specific manner or by heat-stable *phytase* gene, overexpression of *NAS* gene family members like *NAS1* and *NAS2*, incorporating beta-carotene pathway inside rice seed, overexpression of some proteins with high lysine content along with some genes from other organisms, overexpression of some synthetic proteins or by manipulating the storage of prolamins and glutamines proteins and crossing with the high nutrient or protein content rice varieties with high yielding ones to produce high yield nutrient, protein varieties. In this book chapter, we are trying to summarise the previous work and restless effort by the great scientists to achieve the goal through genomic and genetic engineering of rice to provide balanced nutrition.

2 Improvement of Mineral Content in Rice

2.1 Overexpression of *Ferritin* Gene to Increase Iron and Zinc

Iron and zinc are two essential micronutrients for the human diet. Rice grains are deficient with iron and zinc; to improve the iron and zinc in rice grain, Vasconeclos et al. (2003) transformed a soybean *ferritin* gene in seed-specific manner. For that, the fragment of soybean *ferritin* gene (0.8 kb) was amplified from cDNA, and the amplified fragment was ligated onto a *pGPTV* vector placed under the regulation of a seed-specific glutelin promoter (GluB). The vector containing the 0.8 kb

fragment was transformed into a high iron containing line (IR 68144) through the biolistic method of transformation for the development of transgenic plants. Transgenic plant seeds were screened for the estimation of iron and zinc content. Results showed that the iron concentration was significantly increased in high iron rice lines; also the level of zinc significantly increased in the transgenic rice seeds.

2.2 Development of High Iron Rice with Improved Absorption

Iron (Fe) is an essential micronutrient for the human diet, and rice which is considered being a staple food for the human diet contains the least amount of iron in seed. Deficiency of iron is linked to a severe disease is called anaemia. To improve the iron level and absorption in human body, Lucca et al. (2008) performed a work that improved the iron level and intake of iron in the human intestine. In order to increase the iron content of rice, the ferritin gene was cloned from *Phaseolus vulgaris* and transfer it on rice. The phytic acid level also an important factor for iron availability and absorption. The level of phytic acid was decreased in rice through an expression of a heat-stable phytase enzyme cloned from *Aspergillus fumigatus*.

2.3 Enhancement of Iron and Zinc Through Overexpressing the *OsIRT1* Gene

Iron and zinc are the essential micronutrients and both are necessary for the function of numerous enzymes; iron acts as a cofactor for several enzymes. Rice grains contain a limited amount of iron and zinc which is not sufficient for human. To improve the iron and zinc content in rice, Lee and An (2009) overexpressed an iron transporter gene in a constitutive manner to produce transgenic rice lines. There are two homologs of *IRT* family genes named *OsIRT1* and *OsIRT2 in rice*. Therefore, OsIRT1 gene cloned and ligated onto a binary vector *pGA1611* under the regulation of a constitutive promoter (maize ubiquitin promoter) to produce *pGA2857* and finally transferred through agrobacterium method to produce transgenic rice lines overexpressing the *OsIRT1* gene. Further analysis with flag leaves at the reproductive stage shows that transgenic plants are accumulating an excess amount of iron and zinc compared to wild type control plants. When seeds of transgenic plant were analysed for the estimation of iron and zinc content, results showed transgenic plant had significant amount of increase iron and zinc on seeds.

2.4 Improvement of Rice Grain by *Nicotinamine synthase*

Nicotinamine (NA) is the major factor for metal homeostasis which acts as a chelator and is synthesised from s-adenosyl methionine (SAM) by the activity of *NAS* (*Nicotinamine synthase*) gene. To that point of view, it was hypothesised that *NAS*

gene members may also involve in micronutrient homeostasis. Rice seeds are deficient with essential micronutrient like iron and zinc. To improve the concentration of iron, Lee et al. (2008) performed work to overexpress *OsNAS3* gene under the regulation of a constitutive promoter. Therefore, OsNAS3 gene cloned and ligated on a binary vector to produce transgenic rice. *OsNAS3* gene helps to accumulate an increased amount of iron into the rice plant. In order to validate, mutant plant was developed with *Osnas3* gene followed by the transformation of *OsNAS3* gene. Results showed that *OsNAS3* provides tolerance during iron- and zinc-depleted condition and accumulates more iron on seeds. When seeds of the transgenic plant were analysed for the exact amount of iron content through SEC-ICP-MS (size exclusion chromatography-inductively coupled mass spectrometry), it shows transgenic plant seed with an increased amount of zinc and iron.

2.5 Development of High Iron and Zinc Content Rice Through Breeding

In 1992, IRRI (International Rice Research Institute) started to estimate the effect of soil on the iron content of rice grains. As a part of the project, they screened the genotypes and estimate the amount of iron and zinc present on rice seeds. Screening of germplasms showed that common cultivars like IR 64 and IR 72 contain about 12 mg of iron and 25 mg of zinc and some traditional cultivars like Jalmagna, Zuchem and Xua Bue Neo had a higher concentration of iron and zinc in the seed. Popular varieties like IR 64, IR 72 and IR 36 have a limited amount of iron and zinc on seeds. To improve the iron and zinc content in seeds of popular and high yielding varieties, Gregorio et al. (2000) crossed popular high yielding variety IR 72 with a traditional cultivar with high content of iron and zinc (Zua Bondat), from which an elite line was identified (IR 68144-3B-2-2-3) that showed an increased level of iron and zinc on the seed at about 21 ppm (parts per million).

3 Enhancement of Protein and Essential Amino Acid Content

3.1 Improvement of Protein Content Through *Aspartate Aminotransferase*

Enzyme *aspartate aminotransferase* (*AAT*) is essential for amino acid biosynthesis and catalyzes the transfer of α-amino group from aspartate to glutamate. The interconversion of aspartate and glutamate depends on the activity of *AAT*s. Depending on that concept, Zhou et al. (2009) identified four AAT genes of which three genes from rice *OsAAT1, OsAAT2* and *OsAAT3* and one gene from *Escherichia coli* (*EcAAT*). The gene cloned under the regulation of a constitutive promoter (*CaMV35S*) was transformed through *Agrobacterium*-mediated indirect method of transformation technique. Results showed that the transgenic plants overexpressing *OsAAT1, OsAAT2* and *EcAAT* had higher amino acid and protein content

on leaves and seeds than control plants. *OsAAT1, OsAAT2* and *EcAAT* over-expressed transgenic lines showed 16.1, 12.0 and 5.4% higher amino acid content than the control. However, *OsAAT3* lines did not show any significant change in amino acid and protein content.

3.2 Development of High Protein Content Rice by Expression of *Amino Acid Permease*

Grain protein content (GPC) is the main factor to determine the nutritional quality of grains, and rice grain contains least GPC. So, protein and other nutrients have been increased by targeting GPC directly. Depending on that concept, Bo Peng et al. (2014) identified a QTL qPC1 directly related to grain protein content, which regulates the accumulation of amino acids and synthesis of amino acid-like glutelins, prolamins, albumins and globulins. Fine mapping showed that qPC1 was 6.7 kb in size and encoded a putative amino acid permease (*OsAAP6*, 4.7 kb) which directly regulates GPC. The region of *OsAAP6* identified by map-based cloning and ligated on *pCAMBIA 1301S* vector for the development of over-expression construct. RNAi construct was developed through the cloning of fourth exon of *OsAAP6* gene inserted into *dspCAMBIA1301* vector and transformed into plants to generate transgenic lines. Results showed that over-expressed lines accumulated a higher amount of grain proteins (glutelins, albumins, globulins, prolamins) and amino acids like alanine, leucine, arginine, proline and acidic amino acids than control plants.

3.3 Improvement of Essential Amino Acid and Protein Content Through Synthetic Fusion Proteins

Rice seeds are rich in carbohydrates, but lack some essential amino acids like lysine and threonine and protein. So, a protein on seeds may be increased by a synthetic protein which is formed by the fusion of a motif with plant protein. Jiang et al. (2016) showed that seed storage protein in rice was increased by the fusion of two motifs designated TkTKK1 and TKTKK2 with two rice genes to increase essential amino acid content and protein content in rice seed. Thererfore, two genes LOC_Os12g16880 and LOC_Os08g03579 was identified by microarray. LOC_Os12g16880 exhibited seed-specific expression and encoded a seed storage protein, whereas the LOC_08g03579 gene was expressed in multiple tissues, including seeds with unknown function. TKTKK motifs were synthesised by IDT (Integrated DNA Technologies) and amplified using forward and reverse primers with *Bam*HI and *Bgl*II restriction sites. Primarily, the gene was cloned in *pGEM-T Easy* vector, then subcloned again on same *pGEM-T easy* vector by digesting vector with *Bgl*II and amplified fragment with *BamH*I and *Bgl*II restriction enzymes, followed by ligation and transformation to produce 16 times of TKTKK motifs and finally fused with LOC_Os12g16880 assigned as pTKTKK1 and LOC_Os08g03579 assigned as pTKTKK2, separately and a tandem array of two

genes in a construct assigned as pTKTKK3, subcloned into *pCAMBIA 1300* vector under a constitutive promoter CaMV35S and transformed into *Agrobacterium tumefaciens* through electroporation. HPLC analysis demonstrated that amino acids content like lysine and threonine of transgenic seeds from TKTKK1 and TKTKK2 line had increased several fold compared to non-transgenic control. However, TKTKK3-containing seeds showed limited improvement in the seed storage protein and nutrients.

3.4 Development of High Lysine Rice Expressing Lysine-Rich Protein

Rice seeds are deficient with lysine. Therefore, to improve the lysine content in rice seeds, Liu et al. (2016) identified a gene from a leguminous plant, dragon bean, named *Psophocarpus tetragonolobus,* which encodes a protein-rich with lysine. The *LYSINE RICH PROTEIN* (*LRP*) gene encodes an 18 kDa protein. Further, amplify this gene by using gene-specific primers and cloned into a binary vector pBS130 under the control of an endosperm specific promoter Gt1 (GLUTELIN1). A marker *HPT* (*Hygromycin phosphotransferase*) gene under the control of a constitutive promoter CaMV35S was also cloned on the same *pBS130* vector. The complete cassette (*pBS130-LRP*) was transferred into PA64S (Peiai 64S is an elite photoperiod-thermosensitive male sterility, PTSMS) line by *Agrobacterium*-mediated transformation technique. Transgenic plant line PA110 was confirmed by southern blot hybridisation for copy number of T-DNA insertion followed by qRT-PCR and western blot analysis for the expression of exogenous gene. The abundance of lysine content, other amino acids like methionine (Met), cysteine (Cys) and tyrosine (Tyr) and as well as protein was increased significantly in transgenic plants compared to control plants. Lysine content in the endosperm of transgenic plant seed was high, 34.76%. Expression studies of lysine biosynthesis pathway genes like *OsDAPD* were increased in PA110 line, and expression of *OsLKR/SDH* gene which encodes a bifunctional enzyme involving in lysine catabolism was decreased 15 days after flowering stage comparing with PA64S. This study indicated that reduced catabolism of lysine might help in accumulation of more lysine to seeds.

3.5 Increased Lysine Content of Rice by Manipulation of Gene Involved in Aspartate Pathway

Lysine is an essential amino acid which is produced through aspartate pathway with the activity of several enzymes, of which *DHPS* (*Dihydrodipicolinate synthase*) is involved in the production of dihydrodipicolinate for synthesis of DHPS enzyme activity. DHPS is controlled by the level of lysine within the plant (i.e. feedback regulation of DHPS enzyme by lysine). To improve the lysine content in rice seed, Lee et al. (2001) identified a lysine feedback-insensitive dihydrodipicolinate from maize (*Zea mays*), which is not regulated by the level of lysine. The gene was cloned

from maize and two vectors were constructed to check the effects of feedback insensitive *DHPS* within the plant. One construct cloned in a binary vector under the control of a constitutive promoter CaMV35S and another under the control of an endosperm-specific promoter (which especially expressed within endosperm) GluB-1 (GLUTELIN B-1). Finally, both constructs were transferred into the rice plant to generate transgenic lines. Transgenic lines with constitutive promoter and maize-mutated *dhps* referred TC showed an increased lysine levels of seeds significantly. Another line expressing under the glutelin promoter and contained maize-mutated dhps gene did not show any significant change compared to the control (wild)-type plants.

3.6 Improvement of Lysine Level in Seed Through Over Accumulation of BiP Proteins

BiPs are lysine-rich protein that acts as a molecular chaperon and interacts with protein in endoplasmic reticulum (ER) lumen. The ratio of lysine in BiP is higher than glutelins and prolamins. Over-accumulation of BiP protein induces strong E.R stress. To study, what kind of behaviours plant show during over accumulation of BiPs protein, Kawakatra et al. (2010) developed the transgenic lines of rice plants where BiPs protein were over-expressed by using an endosperm-specific promoter and glutelin promoter. Overexpression lines showed a significant increase in lysine content compared to the wild type. In addition to, total amino acid content also increased compared to wild-type plant. The abundance of lysine and tyrosine were increased by 2.7- and 0.4 fold, respectively, in transgenic BiP over-expression lines. Starch content decreased by 48% on transgenic lines, therefore, seed weight also reduced compared to wild-type rice plants. Besides, seed storage protein (SSP) was down-regulated due to over expression of BiP protein, and contents of some non-SSP proteins were increased due to the compensatory effect under stress.

3.7 Development of High Lysine in Rice Through Endogenous Histones

Rice seeds are deficient with lysine and other essential amino acids. To improve the lysine content and nutritionally balanced rice seed, Wong et al. (2015) developed some transgenic lines by over-expressing two endogenous histone proteins, which showed homology with rice H2 family histones *RLRH1* and *RLRH2*. They identified potential candidates gene by searching GenBank database and cloned *RLRH1* (GenBank: Os05g0113900) and *RLRH2* (GenBank: Os01g0502900) from mRNA with using gene-specific primers. Primarily, the amplified fragment was cloned onto a TA cloning vector, followed by subcloning onto the binary vector *pBS130M* under the control of a modified endosperm-specific promoter pmGT1 (modified GLUTELIN1 promoter provides enhanced level of expression in aleurone rather than original GT1 promoter). A total of eight different constructs, were developed of

which, pA1 and pB1 constructs contains *RLRH1* and *RLRH2* genes under the control of modified pmGT1 promoter. pA2 and pB2 constructs were without NLS (Nuclear localization signal) RLRH1-NLS and RLRH2-NLS under the control of endosperm-specific promoter pmGT1 to ensure their expression on cytosol. pA3 and pB3 constructs were with a specialized GT1-SP (GLUTELIN1 signalling peptide), a signalling peptide before *RLRH1* and *RLRH2* genes to ensure the proteins expressed on ER (endoplasmic reticulum) under the control of modified GT1 promoter pmGT1. pA4 and pB4 constructs were with GT1-SP and *RLRH1* and *RLRH2* genes without NLS under the control of pmGT1. Eight vector constructs were used for localisation and expression studies, and results showed that pA1 localised at nucleus, pA2 localised at cytosol, pA3 localised at PSVs and pA4 localised at PSVs, and the results were the same for pB1, pB2, pB3 and pB4. PSVs are one kind of vacuole, unique in plants, and store minerals and proteins essential for germination. Then, constructs were mobilised into *Agrobacterium* EHA105 strain by heat shock method and transformed to rice by *Agrobacterium tumefaciens*-mediated rice transformation technique. Transgenic lines containing pB1 and pB2 failed to germinate due to some adverse effects. From other six constructs, transgenic plant was developed. Six transgenic lines were checked with southern blot and amino acid analysis. The plants were separated depending on high lysine content and low lysine content. High lysine containing line with pA3 constructs showed 34% increase in lysine content in seed followed by transgenic plant line with pB4 constructs with 24% increase in lysine content. pA3 and pB4 transgenic lines with the highest amount of lysine give an indication that targeting endoplasmic reticulum may increase lysine content in seeds. Further analysis also showed that the proteins expressed in pA3 and pB4 transgenic lines were fulfilling the requirements of WHO (World Health Organisation).

3.8 Improvement of Lysine Content in Seeds Through Altered $tRNA^{lys}$ Genes

Lysine is an amino acid encoded by AAA and AAG codon normally in eukaryotes. In 1998, Chenz et al. manipulated *Arabidopsis* $tRNA^{lys}$ genes to generate tRNA, which inserted lysine in place of glutamine, asparagine and glutamic acid and encodes termination codon with lysine and introduced into tobacco to check the result. Depending on that, Wu et al. (2003) produced transgenic rice lines by introducing $tRNA^{lys}$ which inserted lysine with alternate codons during translation. In this study, they developed various constructs pLys/am, which had altered CTT to CTA sequence and seven copies of $tRNA^{lys}$ (CUA) inserted into pBluescript-SK vector with maize ubiquitin promoter (UBI-I) and 5′ untranslated sequence. The $tRNA^{lys}$ (GUU) (Asn), $tRNA^{lys}$ (CUG) (Gln) and $tRNA^{lys}$ (CUC) amplified from ptLys/Asn1, ptLys/Gln and ptLys/Glu DNAs and finally incorporated into *pUC19* vector to generate Tri-tRNA. *Luciferase* gene was modified through the incorporation of a stop codon (UAG) by changing AAG codon present at 206th place. Transfer into rice callus by bombardment to which transgenic rice lines were

developed. The production of intact luciferase indicates ptLys/am insert lysine in place of termination codon. The result of the amino acid analysis showed that the expression of $tRNA^{lys}$ (CUA) which inserted a lysine at the position of termination codon helps to increase the lysine content in prolamin about 43%. The plants had Tri-tRNA genes coding lysine at the position of asparagine, glutamine and glutamic acid, which increased their lysine content in seed about 75% without any significant alterations on seed size and seed total protein content.

3.9 Enhancement of Lysine Content in Seeds Through *Sb401* Protein

Lysine is an essential amino acid which is necessary for human. Rice seeds have the least amount of lysine on seeds. To improve the lysine content on seeds, Dengfeng et al. (2005) transferred a gene into rice from *Solanum berthaultii,* which encoded a high lysine-rich protein and expressed in pollen. For that study, the gene was amplified by PCR (polymerase chain reaction) from mRNA and cloned it on a vector under the regulation of an endosperm-specific promoter to ensure their expression specifically on the endosperm. The cassette was transformed into two types of rice varieties calli derived from japonica (Nippobare cultivar) and indica rice 501R by *Agrobacterium*-mediated transformation. Insertion of gene in transgenic lines was analysed with southern blot hybridisation and PCR technique. Eighteen transgenic lines, of which thirteen from Nippobare cultivar and five from indica rice 501R, were selected for further analysis, of which ten transgenic lines showed an elevated level of lysine and protein on their seeds. The total lysine content was increased at about 20% for transgenic plants. T_0 plant seeds were used for amino acid analysis, which showed total lysine content increased by 20%. Among them, one of the transgenic line showed outstanding performance where lysine content increased at about 35.34% and total protein of the seed increased about 32.74%. From this results, it was concluded that *Sb401* gene efficiently increases the total protein and lysine of seeds when expressed on seed-specific manner.

3.10 Accumulation of Lysine and Protein Content on Rice Seeds by *AmA1* Gene from *Amaranthus hypochondriacus*

Rice is an essential food for a large number of populations across the world. The seed of rice contains the least amount of essential amino acids (EAAs) like lysine, methionine, threonine, etc. To improve the total lysine and protein content on rice seed Xu et al. (2017) performed work based on cloning a gene and transgenic rice plant development. For that, they at first amplified a gene by using PCR from *Amaranthus hypochondriacus*, which is seed albumin and rich in EAAs and cloned the fragment on a binary vector *pCDMAR*. Binary vector was constructed with the gene of interest (GOI) under the control of glutelin-1 promoter and selectable marker gene *HPT* (*Hygromycin phosphotransferase*) under the regulation of a

constitutive promoter from cauliflower mosaic virus. The constructed binary vector was transferred into an elite rice indica variety MH86 by *Agrobacterium tumefaciens*-mediated transformation. Transgenic lines were conformed with hpt and *AmA1* gene-specific primer. Transgenic plants of T_0 generation were confirmed with southern blot hybridisation, and expression of *AmA1* gene was analyzed with qRT-PCR and western blot analysis. The seeds of T_2 generation lines were measured with kjeldahl and high-speed amino acid analyser which showed seven transgenic plants showed significant increase of essential amino acid content compared to control plants. Lysine content increased at about 10.8%; threonine content increased at about 9.1%. Other amino acids also increased on transgenic plants like valine (14.6%), isoleucine (12.1%) leucine (14.7%) and phenylalanine (15.6%).

3.11 Improvement of Cysteine and Methionine Content Through Sesame *2S Albumin* Gene

Methionine is a sulphur-containing essential amino acid which animals are unable to synthesize it, so it needs to be acquired from daily diet. Animals are able to convert methionine to cysteine. Sesame seed (*Sesamum indicum* L.) is enriched with methionine contains *2S albumin*, the major soluble protein in sesame at first synthesised as a 17 KDa protein, then cleaved from N-terminal signal sequence for further processing and folding into the endoplasmic reticulum (ER). Finally, processed and mature sesame protein comprises two subunits 9 KDa and 4 KDa joined by disulphide bonds. To improve, the methionine and cysteine in rice seeds, Tiger et al. (2003) performed a work to increase the amount of sulphur-containing amino acids by generating transgenic rice. For that, the chimeric gene encoding precursor of the polypeptide of sesame 2S albumin (400 bp) cloned under the regulation of a seed-specific glutelin promoter (1200 bp) and transferred into *pCAMBIA 1300* vector between restriction site *Hin*dIII and *Eco*RI to generate *pGlu2S* construct. The cassette was mobilised into *Agrobacterium tumefaciens* EHA105 strain by electroporation. The complete cassette was transferred into *Oryza sativa japonica* cv TNG67 calli, callus developed from the immature embryo and infected with *Agrobacterium tumefaciens* EHA 105 construct containing strain for transgenic plant development. Accumulations of S2SA protein in transgenic seed were checked with western blotting and in situ localization. T_1 generation plants further planted on the greenhouse, and from that T_2 generation arises of which five lines showed complete homozygous nature and of which one best transgenic line is subjected to crude protein and s-amino acid analysis. Results showed that protein content of T_2 generation rice seed had increased about 0.64–3.54%. The best line of T2 generation seed demonstrated a significant increase of methionine and cysteine content comparing to control plants.

3.12 Improvement of Rice Grain by Targeting 13 kDa *Prolamin* Gene Through RNAi

Rice seed contains two types of protein bodies (PB-I and PB-II). PB-I is indigestible and stores prolamins mainly. Prolamins are stored in the form of spherical protein bodies and are of three types such as 10 KD, 13 KD and 16 KD. PB-II mainly contains globulin and glutelins type of proteins. To improve the essential amino acid and other protein, Kim et al. (2013) performed a work to target the gene through RNAi and suppressed the expression of 13 KD prolamin gene. For this experiment, a partial fragment of 13 KD *prolamin* gene was cloned from RNA by gene-specific primers, which is 506 bp containing attB1 and attB2 sequence and subcloned with a donor vector *pDONR221* to generate entry clone with the help of BP Clonase. The entry clone and a destination vector *pANDA-B* were used to generate expression vector with the help of LR Clonase. The gene of interest was cloned here by gateway cloning procedure. The binary construct was transformed into *Agrobacterium* strain, and finally the construct was transferred into rice through *Agrobacterium*-mediated transformation to generate transgenic lines. Transgenic plants were checked with qRT-PCR, SDS-PAGE and immunoblotting. The results showed that activity of 13 KD gene was suppressed by the RNAi construct. The amino acid analysis also showed lowered amount of 13 KD prolamin protein, and other essential amino acids were increased except glutamic acid as a compensation effect of prolamin. Remarkably, one of the essential amino acid, lysine, content was increased significantly (28%) in transgenic rice comparing with wild type. Therefore, this study indicated that the nutritional content and protein content in rice seed might be increased by suppression of prolamin by RNAi approach.

4 Improvement Through Increasing the Resistant Starch

4.1 CRISPER/Cas9-Mediated Genome Editing of Starch Branching Enzymes

Starch is a polysaccharide made up of glucose monomers, which are joined by α-1, 4 linkage. There are two forms of starch amylose and amylopectin. Amylose is a linear form of starch, and amylopectin is a branched form of starch. Plant stored starch as a major energy source, of which 20–25% are amylose and 75–80% is amylopectin. Amylopectin is an easily digestible form than amylose. Resistant starch is a form of starch which constituents are not digestible and contains high amount of amylose than amylopectin thus helping to maintain the glycaemic index of blood, also helpful for type II diabetes. When rice is cooked, the amylose form of starch re-associates rapidly on cooling rather than amylopectin which resists digestion. To improve the amylose content on rice, Sun et al. (2017) developed a high amylose rice line through genome editing of starch branching enzymes by Clustered Regularly Interspaced Pallindromic Repeats (CRISPER)/CRISPER-associated 9 (Cas9). CRISPER/Cas9-mediated genome editing helps to edit gene specifically by targeting

the specific positions. For that, *SBEIIa* and *SBEIIb* were identified, which involved in starch branching. *SBEIIa* and *SBEIIb* showed 80% sequence similarity and *SBEIIb* predominantly expressed in endosperm. To edit the sequences of *SBEI* and *SBEIIb,* gRNA1 (target *SBEI*) and gRNA2 (target *SBEIIb*) were constructed with the help of overlapping PCR and placed them under the regulation of a promoter U3. The gRNA1 and gRNA2 cassettes were ligated on *pCXUN-Cas9* vector and transferred onto the *Agrobacterium tumefaciens* EHA 105 strain by electroporation and finally infected the embryo calli of rice through *Agrobacterium*-mediated transformation technique. Transgenic plants were screened with PCR/RE (polymerase chain reaction/restriction digestion) assay. The basic principle is when Cas9 protein mutates the restriction site, the restriction endonuclease is unable to cut the specific site, therefore allowing the PCR primer to bind. If not, then the restriction endonuclease cut the DNA at a particular site, and there will be no more any site for primer binding. DNA was isolated from the control and transgenic plant and allowed for restriction digestion followed by PCR. The plants which showed positive PCR products were positively edited by the Cas9 protein and activity of DNA damage repair. PCR products then ligated onto a TA cloning vector and sequenced to identify the nucleotide changed. The PCR/RE assay and sequencing result showed that some plants are homozygous, some heterozygous and some biallelic lines. Further analysis of starch contents showed that *sbeIIb* mutant line had higher amount of amylose and resistant starch compared to wild type and *sbeI* mutants.

4.2 Improvement of Grain Content Through Reduction of Amylose Content

Gluten protein is completely absent in rice grains; rice is a gluten-free food which provides health benefit for the people who are suffering from coeliac diseases. Gluten is a family of protein found in grains of wheat, barley and rye. Gluten proteins are of two types gliadin and glutenin. Reports showed that gluten-free diet is favourable for the personal with coeliac disease but unhealthy or has some negative impact of common people. Gluten proteins have some positive effect on common people who are not suffering from coeliac disease, like it reduces the chance of heart attack and acts as a probiotic, feeding some good bacteria of the human body. Grains of rice enriched with starch, specifically amylose and amylose content of rice grains are controlled by function and activity of a dominant *Waxy* gene. Waxy gene function related with the amount of amylose present on rice. The *Waxy* gene encodes a granule bound starch synthase (GBSS) known as Waxy protein which plays a role in amylose synthesis. Studies showed that indica rice contains higher amount of amylose than japonica rice cultivars. The reason behind is allelic variation between indica and japonica rice; indica rice have *Wx a* type of allele, and japonica rice have *Wx b* type of allele with a single base pair on chromosome G/T. This provides messenger RNA stability on indica rice cultivars and tenfold more expression than japonica rice. To improve the grain nutritional quality and reduce the amylose content on rice, several works had been performed.

Of which, recently Zhang et al. (2017) published a work to generation of glutinous rice by genome editing and targeting a dominant gene (*Waxy*) in amylose synthesis and converted two japonica non glutenious variety to glutenious variety. In this experiment they used two elite variety japonica plant lines Xinshui 13 (XS134) and Wuyunjing 7 (9522) to reduce the amylose contain and improve the glutelin protein. For that, they developed gRNA with the help of CRISPER-P software which targeting the *Waxy* gene at position of 244–263 inside the exon and clone the gRNA (gRNA was 19–20 bp long with a PAM {protospacer-associated motif} recognition site) at first on a gRNA expression vector then binary cloned on a vector containing Cas9 protein under the regulation of a constitutive promoter and NLS (Nuclear localization signal) inside T-DNA right and left border and finally transferred onto a *Agrobacterium tumefaciens* EHA 105 strain. The embryo calli of both japonica rice cultivars were infected with *Agrobacterium,* followed by the selection and regeneration of plants. The plants that survived were further tested with PCR-based method and sequencing by the sanger sequencing method. Homozygous plants of XS134 and 9522 varieties were identified and results showed that amylose contain was decreased on the plants having mutated waxy gene. The low AC in the CRISPR-waxy seeds qualifies them as waxy or glutinous or sweet rice. Transgenic plants were checked with other agronomic traits, which revealed no negative effect on yield if plants contains a mutated waxy gene.

5 Improvement Through Essential Fatty Acids

5.1 Improvement of Rice Grain Through an Expression of *Fatty Acid Desaturase* Gene from Soybean

Fatty acids both saturated and unsaturated are essential for the human body. There are some essential fatty acids that the body is unable to produce and therefore must be available through food. These essential fatty acids are linolenic acid (omega 6 group) and alpha linolenic acid (omega-3 group), which help to absorb some nutrients and plays a role in the production of some hormones. Rice seeds contain little amount of fatty acids, which are not sufficient for the human body. Fatty acids mainly that belong to the omega-3 group (alpha-linolenic acid) are essential for some vitamins and help to protect heart attack, control hypertension, etc. To improve the alpha-linolenic acid in rice seed, Anai et al. (2003) developed a transgenic rice line that expresses an omega-3 fatty acid desaturase gene from soybean. The omega-3 fatty acid desaturase gene amplified from cDNA with the help of gene-specific primers and cloned the fragment of coding *FAD3* gene and placed under the regulation of a constitutive maize ubiquitin promoter (Ubi-1). The *FAD3* gene helps in the production of an omega-3 fatty acid and alpha-linolenic acid. The complete construct was transformed to *Agrobacterium tumefaciens* and inserted into rice genome. The fatty acid analysis showed an increased amount of alpha-linolenic acid contents. Additionally, T_1 plants showed about tenfold higher ALA content in seed.

5.2 Improvement of Rice Grain Through the Expression of Soyabean and Rice *Omega-3 Fatty Acid Desaturase* Gene

Long-chain polyunsaturated fatty acids are very important for human body, which helps to maintain cholesterol synthesis, eicosanoid, etc. The ratio of omega-6 and omega-3 are also important for the human body. Amount of linolenic acid (LA) and alpha-linolenic acid (ALA) in the body maintains various processes; ALA is a precursor of the most important omega-3 fatty acids like eicospaentaenoic acid (EPA), docospenttaenoic acid (DPA) and docosahexaenoic acid (DHA). The human being is not able to convert LA into ALA due to lack of *omega-3 fatty acid desaturase,* which helps to convert LA into ALA. In plants, desaturation of LA to ALA happens on plastids and endoplasmic reticulum. To improve the amount of omega-3 fatty acids (ALA) content in rice seed, Liu et al. (2012) performed work to express *FAD3* genes under the control of an endosperm-specific promoter. Rice contains three copies of *FAD* gene, but their expression is mainly high in the root and very little on seed and leaf. In this study, they cloned the three rice *omega-FAD* genes *FAD3*, *FAD7* and *FAD8* and three soybean FAD genes *FAD3-1, FAD3-2* and *FAD3-3* on a *pMD18-T* vector. Finally, all genes were subcloned in binary vector containing endosperm-specific promoter GluC and transformed into *Agrobacterium tumefaciens*. The calli of rice line were infected with *Agrobacterium* containing construct either *OsFAD3* or *GmFAD3-1*. Transgenic plants were named as GG for *GluC/GmFAD3-1* and GO for *GluC/OsFAD3* depending on the gene transformed on rice. Transgenic plants were screened with northern blot, southern blot and western blot hybridization. Results of northern blot hybridization showed that the expression ALA was significantly higher on transgenic plants rather than non-transformed rice lines. Estimation of amino acid revealed that the amount of ALA was 15.7-fold and 20.0-fold higher than non-transformed lines. Thus ALA content was significantly enhanced in rice seed by transforming both of constructs (*OsFAD3* and *GmFAD3-1*) when expressed under the regulation of endosperm-specific glutelin protein promoter GluC.

6 Development of Nutrient Content Through Lowering Phytic Acid Level

6.1 Improvement of Rice Grain Through the Repression of *1D-Myo-Inositol-3 Phosphate Synthase Gene* (*RINO1*)

Phytic acid (PA) is the major storage form of phosphorous in plant seeds. The enzyme *1-D-myoinositol-3-phosphate synthase* (*RINO1*) catalyzes the first step of myoinositiol and helps the biosynthesis of phytic acid in seed. Phytic acid strongly binds with other mineral cations like potassium, calcium, iron and zinc to form phytate, which is not easily digested by monogastric animals. Phytate is a chelator that also binds with several essential micronutrients, therefore, it blocks the availability of micronutrients and sometimes binds with several enzymes in the seed. To

improve the nutritional content in rice, Kuwano et al. (2009) performed work to repress the activity of *1-D-myoinositol-3-phosphate synthase* gene, which directly helps the biosynthesis of phytic acid. In this experiment, antisense cDNA was amplified by using PCR and product cloned into the binary vector *pGPTV-35S-HPT*. The amplified fragment is 1844 bp long and placed under the regulation of a tissue-specific Ole18 promoter, which specifically expressed on the endosperm. The complete cassette was transformed into *Agrobacterium tumefaciens* EHA 105 strain by electroporation. The embryo calli of rice were then infected with *Agrobacterium* and transformed with *Agrobacterium*-mediated transformation. Transgenic plants were selected by hygromycin resistance and tested with southern blot analysis. The seeds of transgenic rice plants analysed to check the inorganic phosphate and the level of phytic acid by Chen's reagent and ion chromatographic analysis. Further, the transgenic plants were tested by western blot to ensure the amount of enzyme (*RINO1*) produced in transgenic lines. Transgenic plants were tested for four generations, and results showed that phytic acid biosynthesis decreased about 68% in transgenic seeds compared with non-transformed plants. Transgenic plants also showed an increased inorganic phosphate level, which indicates that the amount of free Pi generated by compensating the amount of phytic acid in seed.

6.2 Improvement of Rice Grain Through the Repression of *Inositol 1,3,4,5,6-Pentakisphosphate 2-Kinase* Gene (*IPK1*)

Phytic acid is a significant source of phosphorous in seed and not utilized by monogastric animals due to lack of phytase enzyme. Phytic acid biosynthesis is regulated by several enzymes of which *inositol 1,3,4,5,6-pentakisphosphate 2-kinase* gene (*IPK1*) helps at last step of phytic acid biosynthesis. Phytic acid mainly binds with micronutrients like iron, zinc, calcium and others to form phytate. Seed micronutrient content and inorganic phosphate content can be improved by reducing the level of phytic acid in seed. To improve the micronutrient content and lower the level of phytic acid, Ali et al. (2013a) downregulated the gene involved in phytic acid biosynthesis through RNAi (RNA interference). For this experiment, the gateway cloning method was used to generate the construct, amplifying the gene of interest with the help of gene-specific primers and cloned the gene into an entry vector *pENTR-D TOPO* vector. The fragment was subcloned with a destination vector *pIPKb006* to generate expression vector with the help of LR clonase generate antisense RNA, which further silence the *IPK1* gene. In between, a wheat intron RGA2 was placed for loop formation. Then Ole-18 promoter ligated on this vector by *SpeI* and *HindIII* sites to generate *pOle-18-IPK1-006* vector. The complete cassette was introduced into Pusa Sugandhi II embryo calli with the help of particle bombardment to transfer the expression vector containing the gene of interest under the regulation of a tissue-specific promoter Ole18. Further, transgenic plants were selected by hygromycin resistance and analyses with southern blot and PCR amplifying the intron fragment of wheat RGA2. T_4 generation seeds were allowed to test with total inorganic phosphate, phytic acid content, myo-inositol content and

metal and amino acid content. Results showed that inorganic phosphate was increased on transgenic seed and HPLC (High performance liquid chromatography) data also showed that transgenic plants had reduced level of phytic acid. When transgenic seeds were allowed for AAS (atomic absorption spectrometry) assay for the determination of micronutrients, it shows iron, zinc, magnesium and calcium level increased compared to non-transgenic plants. Amino acid analysis results showed the transgenic and control seed had the same amount of amino acids than non-transgenic. Thus it is clear that the downregulation of *IPK1* did not alter the other essential metabolic processes of rice plant.

6.3 Improvement of Rice Grain Through the Repression of *Myo-inositol-3-phosphate Synthase* (*MIPS*) Gene

Phytic acid is considered the primary source of phosphorous in cereal grains. The reduction of phytic acid is desirable to seeds due to antinutrient properties. Phytic acid chelates with various nutrients to form phytate which was not easily digested by humans and other monogastric animals due to lack of phytase enzyme. To reduce the phytic acid content inside the seed, Ali et al. (2013b) performed a work to reduce the expression of a *myo-inositol-3-phosphate synthase* (*MIPS*) gene through- RNAi (RNA interference), which catalyzes the first step of phytic acid biosynthesis and also essential for myo-inositol formation. For that experiment, *MIPS* gene was amplified using gene-specific primers and clone the gene onto an entry vector *pENTR-D-TOPO* vector. After that, the gene fragment was subcloned in sense and antisense orientation in then destination vector *pIPKb006* by LR clonase. In this study, Ole18 promoter was used to ensure seed-specific suppression of *MIPS* gene expression. Then the construct was transformed into embryo Pusa Sughandhi II rice through biolisticmethod of direct gene transformation method. The transgenic plants were selected against hygromycin resistance and by positive PCR amplification of RGA2 intron. Transgenic plants were then allowed for southern blot analysis; qRT-PCR was performed from transgenic lines to identify the level of reduction of *MIPS* gene. T_3 generation seeds were allowed for phosphate, phytic acid and metal analysis. The results showed that phytic acid accumulation reduced significantly on transgenic seeds and metal analysis through the atomic absorption spectrometer. Subsequently, the iron concentration was significantly increased in transgenic seed compared to non-transformed rice plants.

7 Reduction of Cadmium Content in Rice Seed

7.1 Improvement of Rice Grain by Targeting and Knockout a Cadmium Transporter Gene Through CRISPR/Cas9

Cadmium is highly toxic for the human body. Continuous uptake of cadmium (heavy metal) can cause severe kidney damage, itai-itai disease, cancer and other health problems. Rice plants accumulate a significant amount of cadmium inside plant shoot, root and grains. Previous reports showed that rice grains also contains a large amount of cadmium and cadmium level is high in *indica* rice varieties plant rather than *japonica* rice. To date, several transporters had been identified that transport and accumulate cadmium. *OsIRT1* and *OsIRT2* (*IRT – Iron regulated transporter*), which are the iron transporter also transport cadmium and some members of *NRAMP* family (natural resistance associated with macrophage protein) like *NRAMP5* and *NRAMP1* which also help to uptake cadmium by root. *OsHMA3* helps to transport cadmium into vacuoles of root cells and *OsHMA2* involved in xylem loading whereas *OsLCT1* involved in transferring cadmium from xylem to phloem. To reduce the cadmium content in rice, Tang et al. (2017) knocked out a cadmium transporter *OsNRAMP5* by an advanced method of genome editing of modern biotechnology, CRISPR/Cas9-mediated genome editing. For that experiment, they at first identified a gene that did not affect yield if edited by the above approach. They developed two sgRNA *OsNRAMP5-PS1* and *OsNRAMP5-PS2* targeting the ninth exon of *OsNRAMP5* gene at two positions which are 119 bp apart in between ninth exon of *OsNRAMP5* gene and cloned on a pYLgRNA expression vector under the regulation of rice specific OsU6 and OsU3 promoter which present in between two BsaI restriction site. The *OsNRAMP5-PS1* and *OsNRAMP5-PS2* contains a PAM sequence then 19–20 bp sequence complementary with target sequence which is edited by Clustered Regularly Interspaced Short Pallindromic Repeats (CRISPR)/CRISPR-associated 9 (Cas9). *BsaI* is a type II restriction endonuclease which is used for directional cloning and golden gate cloning. The binary vector *pYLCRISPR/Cas9Pubi-H* was used to develop moncot and dicot plants with codon optimised Cas9 often called Cas9P. The sgRNA was cloned by cutting the vector with *Bsa*I enzyme and discarding ccdB gene situated in T-DNA right and left borders and placing sgRNA from *pYLgRNA* vector, targeting *OsNRAMP5* gene on rice. The whole construct transformed into *Agrobacterium tumefaciens* EHA 105 strain. Two wild-type *indica* varieties of rice Huazhan (HZ) and Longke 638S (638S) were allowed for transformation. The embryo callus was used for Agrobacterium-mediated transformation. T_0 plants were confirmed by PCR-based method by isolating the genomic DNA from transgenic plants, followed by PCR with target-specific set of primers. Amplified fragment was sequenced by Sanger sequencing method to identify the edited part of the gene by CRISPR/Cas9 and identify the homozygous, heterozygous and biallelic lines of transgenic T_0 plants. Results showed that the mutant plant, which had edited or mutated *Osnramp5* gene, reduced cadmium content significantly. The edited line comprised 0.05 mg/kg

cadmium, which is less than the wild type *indica* rice cultivars having 0.33 mg/kg to 2.90 mg/kg cadmium on grain.

7.2 Improvement of Grain Through Targeting *OsNramp5* and *OsLct1* Gene by CRISPR/Cas9

Cadmium, a toxic, heavy, non-essential metal for human body that is transported in the body through the food chain. Continuous uptake of cadmium creates several types of diseases in the human body. Reports showed that when the soil contains a high amount of cadmium, rice plant also accumulate a significant amount of cadmium inside shoot, root and grains. To reduce the cadmium content in rice, various works had been performed by various scientists of which Songmei et al. (2019) performed work to develop rice lines where *OsLct1* gene or *OsNramp5* gene mutated by targeting the gene through CRISPR/Cas9. Lct1 is a low-affinity cation transporter one identified by homology search from wheat and involved in cadmium transport. Nramp5 is a cadmium transporter which accumulate significant amount of cadmium into rice plant. To develop low cadmium rice lines, *OsLct1* and *OsNramp5* gene was knocked down by sgRNA that targeting exon 1 for *OsLct1* (named *Oslct1∗1*) and exon7 (named *Osnramp5∗7*) and exon 9 (named *Osnramp5∗9*) for OsNramp5 gene. sgRNA was developed with the help of CRISPR-P programme. sgRNA was cloned into *pHun4c12S* plasmid harbouring a *CYP81A6-hpRNAi* element. Three CRISPR/Cas9 plasmids were mobilized into *Agrobacterium tumeficiens* strain separately and used to infect embryo calli of japonica rice cultivar 'Xidao1' to generate transgenic rice lines. The mutant plants had accumulated 13.7% less amount of cadmium into seeds compared to non-transformed 'Xidao1' line rice plants. No significant alteration was observed in total grain numbers and panicle number per plant when compared with non-transformed plant. The transgenic lines mutated with *OsNramp5* gene also showed significant reduction of cadmium content compared to non-transformed lines plant.

8 Development of Transgenic Rice with Reduced Arsenic Accumulation by Overexpression of Nodulin 26-Like Protein

Arsenic is a toxic element that enters the human body through water and food. Rice grains contain higher amount of arsenic compared with other cereal crops. As (III) which is predominant form of arsenic has similar physiochemical properties with silicic acid therefore transported by the silicon transporters like *OsLsi1* (*Low silicon 1*) and *OsLsi2* (*Low silicon 2*). As (V) is a chemical analog of phosphate and transported by the phosphate transporters like *OsPT1*, *OsPT4* and *OsPT8* in rice. Within plants there is some As (V) reductase, which reduced to form As (III). Plants detoxify arsenic into the external medium by several processes and in the cell by chelating with phytochelatins. To improve the grain quality and reduction of arsenic

several works had been performed like knocking out *OsLsi2* gene, which significantly decreases the amount of arsenic in grains but reduced silicon uptake and reduced grain yield. Knocking out phosphate transporters also affect plant growth. To reduce the arsenic content in grains without affecting other essential nutrients and parameters, Sun et al. (2018) performed work to overexpress two aquaporin genes *OsNIP1;1* and *OsNIP3;3*. For this experiment, they at first cloned the ORF (open reading frame) of *OsNIP1;1* (855 bp) and *OsNIP3;3* (837 bp) from the cDNA and cloned them under the control of a constitutive maize ubiquitin promoter to *pTCK303* and *pAL103* vectors and then subcloned onto *pCambia1302* vector. To identify the site where two proteins *OsNIP1;1* and *OsNIP3;3* are localized, the genes were tagged with *pTCK303-FLAG* vector. *OsNIP1;1* FLAG containing sequence is also cloned under the regulation of a tissue-specific promoter *pOsLsi1* (promoter sequence of *OsLsi1* gene) and cloned onto *pTCK303-FLAG* vector. Knockdown mutants of both *OsNIP1;1* and *OsNIP3;3* were generated by developing and synthesis of sgRNA (single guide RNA) which targeting the first exon of *OsNIP3;3* and second exon of *OsNIP1;1* cloned under the control of U3 promoter. Further, the sgRNA cloned onto *pOs-Cas9* vector by gateway cloning method using LR clonase. The constructs were then mobilized onto Agrobacterium for transgenic development. Subcellular localization of *OsNIP1;1* and *OsNIP3;3* genes were checked with by cloning the gene on *pSATA-eGFP* vector under the regulation of a constitutive promoter and transfecting protoplast of rice by PEG (polyethylene glycol)-mediated transformation or in tobacco (*Nicotiana benthamiana*) leaf epidermal cells by *Agrobacterium*-mediated transformation. Results showed that knockout of *OsNIP1;1* and *OsNIP3;3* through CRISPR/Cas9 did not affect arsenic accumulation. Overexpression of *OsNIP1;1* and *OsNIP3;3* reduced arsenic concentrations in shoots compared to wild types, but there was no significant result for arsenic concentrations in roots. The amount of arsenic content significantly decreased on shoots of the transgenic lines overexpressing *OsNIP1;1* and *OsNIP3;3*. Transgenic plants also showed lower arsenic concentrations on rice grains comparing with wild type plants. Transgenic plants showed no significant difference with other essential nutrients. Critical analysis showed that overexpression of *OsNIP1;1* and *OsNIP3;3* decreased the amount of arsenic concentrations on xylem sap and prevent loading of arsenic inside xylem by leaking out arsenite to the stele. Thus, the molecular approach restricts the arsenite loading in the xylem and prevents the overall accumulation of arsenic.

9 Development of High Carotenoid Rice

Vitamin A deficiency is associated with night blindness, which further leads to total blindness. Beta carotene, a precursor vitamin A is completely absent in rice seed, because inside the rice seed no beta carotene is produced naturally. To produce beta carotene or vitamin A, Ye et al. (2000) produced beta carotene rice. For that they develop several constructs and transformed into rice. Clone *PSY* gene (*phytoene synthase* from *Narcissus pseudonarcissus*) under the regulation of seed-specific Gt1

(Glutelin) promoter and another gene *CRT1* (from *Erwinia uredovora*) under the regulation of constitutive (CaMV) promoter and ligated onto the same vector *pB19hpc*. Further, cloned CRT1 under the regulation of Gt1 promoter and *PSY* gene under the regulation of CaMV promoter and ligated on a same vector called *pZPsc*. Then *LCY* (*lycopene cyclase*) gene under the regulation of *Gt1* promoter named *pZLcyH* vector. The *pB19hpc* vector transformed through Agrobacterium mediated method into rice separately. Where transgenic seeds were red coloured. *pZLcyH* and *pZPsc* vector were transformed into the same *Agrobacterium* strain for co-transformation, and when rice is infected with these *Agrobacterium*, transgenic plants produce beta carotene in seeds and golden coloured.

10 Conclusion

This book chapter summarises the work done by the scientists to improve the nutritional content in rice grain through conventional breeding and modern biotechnological approaches including the overexpression of genes and by genome editing CRISPR-Cas9 (Fig. 1). Improvement of rice grain quality is a basic need due to a large number of poor people whose diet solely depend on rice. They are affected with hidden hunger because rice grain not contains sufficient amount of proteins, fatty acids, iron and zinc. Rice which is considered a staple food all over the world must contain the minimum amount of all essential nutrients and proteins which are necessary for the human diet. Various works have already been done in this field for the improvement of nutritional content, but large work still to do in that field. The goal to achieve a variety of rice which is superior and sufficient to provide all the essential micro and macro-elements for the human still does not exist. Transformation of a gene may affect the plant production, it's really a challenge to make transgenic without affecting its normal traits. It is difficult indeed, but not impossible. Sometimes silencing of a gene might improved the overall nutritional quality of rice but can be severe for the plant and directly affect plant responses to various kinds of stresses, both biotic and abiotic. Breeding, biotechnological and crop improvement divisions of various institutions are constantly working in this field to improve the quality of rice grain, which possibly provides a super rice variety a day which gives all the nutritional, protein and other essential elements.

11 Future Perspective

- Transformation of three genes *OsAAP6, PtLRP (LYSINE RICH PROTEIN)* from *Psophocarpus tetragonolobus* and feedback insensitive *DHPS* (*Dihydrodipicolinate synthase*) on a high yielding high protein rice line and study the difference.
- Overexpression of *OsFER1* and *OsAAP6* gene in seed-specific manner may improve the iron, zinc and proteins in seed.

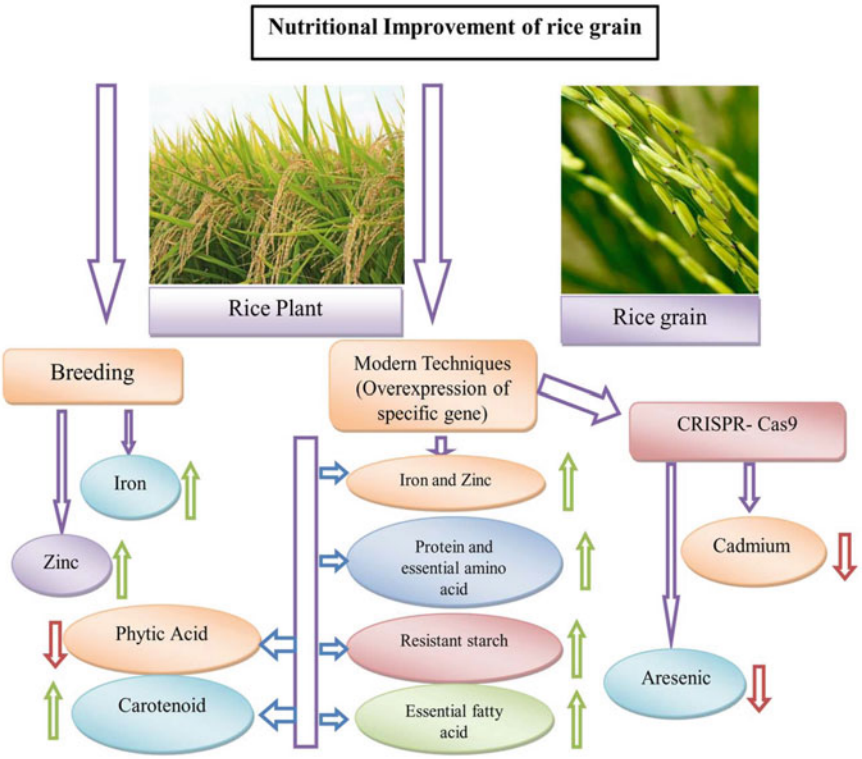

Fig. 1 Nutritional improvement of rice through various approaches, where upward arrow sign means increment and downward arrow sign means decrement

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Genetic Engineering of Rice to Fortify Micronutrients

Aryadeep Roychoudhury and Rituparna Bhowmik

Abstract

Rice, the staple food for most of the Southeast Asian countries, is poor in most of the micronutrients which coupled with the lack of supplementary sources, and poverty leads to severe malnutrition. Fortified rice could be an important alternative to overcome the micronutrient deficiency faced in most the third-world countries. This chapter focuses on genetic engineering as a means of fortifying rice with several vitamins, minerals and amino acids. Earlier works have attempted genetic engineering to improve vitamin A, folate, thiamine (vitamin B1), iron, zinc, lysine, methionine, cysteine and glycinin content of rice. In most of these cases, the responsible genes that affect the specific micronutrient biosynthesis and overaccumulation have been overexpressed under rice seed-specific promoters like *glutelin*. The transcripts for these transgenic plants have been compared, and finally concentration of the micronutrients before and after milling has been recorded. Reasonable success has been seen in most cases, while it also helped to decipher some previously unknown intricate metabolic pathway interactions. This gives us a hope that when consumed in adequate amounts, fortified rice could supply required amounts of micronutrients and prevent deficiency diseases.

Keywords

Fortification · Transgenic rice · Micronutrients · Malnutrition · Transformation · Overexpression

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1 Introduction

A severe health problem affecting billions of people worldwide is 'malnutrition'. More prevalent in the developing or the so-called third world-countries, malnutrition and micronutrient deficiencies are common in all age groups especially young children and women of reproductive age (Rice fortification: its potential for improving micronutrient intake and steps required for implementation at scale, food and nutrition bulletin. December 2012). The statistical figure of such deficiencies stands at:

- Two billion people suffer from iron deficiency (Zimmermann and Hurrell 2007).
- Almost two billion suffer from iodine deficiency (Rice fortification: its potential for improving micronutrient intake and steps required for implementation at scale, food and nutrition bulletin. December 2012).
- 190 million kids and 19 million pregnant women suffer from vitamin A deficiency (World Health Organization 2009).
- 1.6 billion people worldwide suffer from anaemia (De Benoist et al. 2008).
- Deficiencies of micronutrients like folate, zinc and vitamin D are also on the rise (Eichholzer et al. 2006; Sayed et al. 2008).

Micronutrient deficiencies may lead to birth defects (Sayed et al. 2008), retarded growth, slow physical and cognitive development and increasing chances of mortality (Allen et al. 2006). Under such circumstances, scientists are making efforts to fortify rice (a staple food to billions) with micronutrients to compensate these micronutrient deficiencies among masses. In this chapter, some such fortification attempts, using genetic engineering, have been discussed.

2 Why Is Rice a Suitable Choice for Fortification?

About half of the world's population and most of the Asian countries consume 440 MT rice annually as their staple food (US Department of Agriculture 2012). While rice has sufficient amount of vitamins like B1, B2 and B3, most of these are lost during processing steps like de-hulling, milling, washing, etc. Rice forms nearly 61% of the daily calorie intake (Food and Agriculture Organization 2009); the lack of diversity in food choices combined with low micronutrient content of processed rice leads to the risk of developing several micronutrient deficiencies.

Among people of countries like Bangladesh, Lao People's Democratic Republic, Cambodia, etc., severe deficiencies of vitamin A, thiamine, riboflavin and iodine have been seen. These countries face prevalence of anaemia, beriberi and stunted growth among children below 5 years of age. In the rice-consuming countries, above 40% of children below 5 years of age suffer from stunted growth (De Benoist et al. 2008; Dexter 1998; UNICEF 2012; World Health Organization 2009). Studies showed that micronutrient insufficiency from thousandth day of conception to an age of 2 years leads to stunting. This is used as an indicator of micronutrient

malnutrition. Thus, increase in the micronutrient content of rice would make daily micronutrient consumption levels higher. This may help combat the severe situations (The Lancet Special Series 2008).

3 Rice as a Vehicle for Fortification

A food product which is fortified must have some qualities for its socio-economic accessibility; it must be reasonably cheap, regularly eaten, palatable and culturally accepted by the target population. The food item and its fortified nutrient must also be stably maintained under locally available storage conditions (OMNI/Roche/US Agency for International Development 1997). Rice meets most of these requirements; additionally new techniques ensure that the genetically modified rice is indistinguishable from non-fortified rice, increasing its potential of social acceptability.

Types of fortifications in rice:

- Dusting—Dusting on mature grains with micronutrients in powder form.
- Coating—The rice kernel is coated with several layers of the micronutrient mixture made with liquid and waxes.
- Cold Extraction—Dough is made from rice flour or low-quality broken rice, and it is mixed with water and fortified micronutrient. Then the mixture is passed through a pasta press to give fortified rice.
- Hot Extraction—Dough of rice flour, water and micronutrient mix is exposed to steam. This employs a single or twin screw extruder.
- Genetic engineering—Transgenic rice is developed by introducing genes from other organisms to overexpress the specific micronutrient in rice endosperm (Alavi et al. 2008).

4 Fortification in Rice Using Genetic Engineering

4.1 Vitamin Fortification

4.1.1 Vitamin A Fortification

Probably, the best example of vitamin fortification known in rice is for vitamin A, which led to the production of the golden rice expressing functioning provitamin A, i.e. β-carotene biosynthetic pathway in rice. This was possible as transformation techniques in rice are well established; the entire β-carotene biosynthetic pathway in rice is also studied.

In wild-type rice plants, geranylgeranyl diphosphate (GGDP), a precursor of provitamin A, is produced. However, in wild-type plants, this is not directed completely toward carotenogenesis. Expression of four heterologous enzymes of β-carotene pathway, namely, phytoene synthase (psy), phytoene desatuase, ζ-carotene desaturase and lycopene β-cyclase, is necessary. To simplify the

transformation effort by reducing number of transgenes, bacterial carotene desaturase could be used. This suffices as a desaturase for all the four double bonds is required (Burkhardt et al. 1997). Co-transformation of cDNA coding for *phytoene synthase* (*psy*) and *lycopene β-cyclase* (*β-lcy*) from *Narcissus pseudonarcissus* was performed (Burkhardt et al. 1997). The genes were expressed under the control of strong endosperm-specific *glutelin* promoter together with bacterial *carotene desaturase* (*crt1*) which was taken from *Erwinia uredovora* (Beyer et al. n.d.). Three transformation vectors were initially constructed. The vector pB19hcp that expressed phytoene synthase, originating from daffodil, in combination with bacterial carotene desaturase from *Erwinia* under control of Gt1 (glutelin) and CaMV35S promoters respectively is expected to express lycopene in the endosperm of rice from the GGDP (Beyer et al. n.d.). Two vectors, pZPsC (similar to pB19hcp, without the selectable marker) and pZCycH (carrying the cassette for expression of lycopene β-cyclase under *glutelin* promoter) complete the β-carotene biosynthetic pathway. A functional transit peptide was fused with all the genes in all of these plasmid constructs allowing proper import of the proteins to the plastid, the site of GGDP formation (Al-Babili et al. 1996; Bonk et al. 1997; Misawa et al. 1993; Schledz et al. 1996).

Initially transformation was carried out by particle bombardment in immature rice embryos. However, Southern blot analysis revealed that these transformations showed deleterious integration patterns. This led to the use of *Agrobacterium tumefaciens*-mediated transformation in rice. The entire β-carotene pathway could thus be installed in rice by a single transformation effort (Beyer et al. n.d.). Consistent with expectation, transgenic rice so obtained showed β-carotene expressing yellow endosperm. Additional experimentations revealed that *β-lcy* was dispensable in this route, where *psy* and *crt1* alone could give suitable accumulation of provitamin A in endosperm. This led to the construction of pB19hpc plasmid with phosphomannose isomerase (PMI) as the selectable marker in place of antibiotics like hygromycin, thereby ensuring biosafety issues (Lucca et al. 2001a). Efforts are being made to improve the content of vitamin A as well as to implement safe consumption measures. Golden rice is one of the most socially accepted transgenic products; however, its large-scale production, acceptability and marketing would still require some more prerequisites to be fulfiled.

4.1.2 Folate Fortification

Folate deficiencies in pregnant women are a major problem in developing countries which leads to severe cases of neural tube malformation such as spina bifida in infants and megaloblastic anaemia (Geisel 2003; Li et al. 2003). Folate (tetrahydrofolate polyglutamates or THF polyglutamates) are tripartite molecules having a pterin residue which is synthesized in cytosol from guanosine triphosphate (GTP) as precursor, PABA (para-aminobenzoic acid) part derived from chorismate in plastids and several glutamate residues. During its biosynthesis, both pterin precursors and PABA are imported in the mitochondria where they condense with glutamate residues to form folate.

Thus, logically for genetic fortification of folate in rice, both PABA and pterin pathway precursors may be overexpressed to increase the biosynthesis of folate. Folate fortification in rice endosperm has been performed by overexpressing A*rabidopsis thaliana* genes coding for GTPCHI (GTP–cyclohydrolase I) and ADCS (aminodeoxychorismate) from one T-DNA locus. GTPCHI has a negative feedback control on folate production; thus, there was a natural mechanism to prevent undesirably high accumulation of intermediates. To compare the final folate concentration produced by overexpressing genes of each branch, three vector constructs were designed for plant genetic transformation—the G vector with only cDNA encoding *A. thaliana* GTP–cyclohydrolase I, the A vector with cDNA encoding *A. thaliana* aminodeoxychorismate and the AG vector with both these genes expressed on a single T-DNA construct (Nakase et al. 1996; Takaiwa et al. 1991). On transforming Nipponbare japonica variety of rice using *Agrobacterium tumefaciens*-mediated transformation, single copy transformants were screened using Southern hybridization techniques and polymerase chain reaction (PCR). Upon expression of the transformed genes, i.e. *GTPCHI* and *ADCS* with all three of these constructs, the following observations were made.

Transforming construct	Observation
A	PABA levels showed massive increase, up to 49 times that seen in control plant. Surprisingly, folate content here was found to be much lower (six times lower) than control
G	No pronounced increase in folate levels against control; however, 25-fold increase in pterin levels
AG	Massive folate accumulation, i.e. 15–100 times that seen in wild type plants[a]

[a]Plants transformed with empty vectors can also be considered as control. Final accumulation of folate in Nipponbare japonica was in the range of 6.0–38.3 nmol/g (Folate fortification of rice by Nature Biotechnology 2007)

In the transgenic lines, major folate source (accounting up to 89%) was 5-methyltetrahydrofolate. This is more bioavailable than the industrially fortified rice where most of the folate is available as folic acid. High folic acid contents may mask the symptoms of cyanocobalamine deficiency, which is not the case for this transgenic rice. Thus, genetic engineering is a good alternative where industrial fortification is not amenable (Oakley 2002).

One negative feedback loop for folate biosynthesis is due to polyglutamylation. In transgenic variety, only 2.6–14% folates were polyglutamylated in contrast to wild types where it was up to 50% (Melse-Boonstra et al. 2004). This improves the bioavailability of folate, which has been shown to reach a maximum of 38.3 nmol/g. This corresponds to 1723 mg/100 g of fresh weight. This is the highest folate content to be reported in plant world and is far beyond the Recommended Dietary Allowance (RDA) for folate. One setback is instability of folate during cooking; it is estimated to degrade during 30 min of boiling. Thus, a mixed diet is expected to have about

50% of the bioavailable folate. Even so, 100 g of folate fortified rice is expected to meet the RDA for folate making it an excellent choice (Sauberlich et al. 1987).

4.1.3 Thiamine Fortification in Rice

Vitamin B1 or thiamine is essential for synthesizing thymine pyrophosphate (TPP), an important co-factor for various metabolic processes. Populations consuming polished rice are at risk of developing thymine deficiency leading to diseases like Beriberi and Wernicke-Korsakoff syndrome (Joint FAO/WHO Expert Consultation 2004). Women may develop oedema and paraesthesia, while the children are at risk of cardiac diseases, lactic acidosis and gastric disorders leading to increased mortality. Asian population shows a high percentage of women (27–78%) and children (15–58%) affected by thiamine deficiency. Thiamine is mainly stored in staple crops in inedible parts. Thiamine fortification in edible cereal grains may be a way to manage this problem (USDA 2013).

In plants, thiamine is available in the phosphorylated state as mono-, di- and triphosphorylated thiamine, namely, TMP, TDP and TTP, respectively. TTP is the functional form of thiamine; this can be transported in plants and animals only in unphosphorylated forms. Thiamine biosynthesis in *Escherichia coli* and *Arabidopsis* are known in detail via databases like KEGG; major enzyme involved in this pathway includes HET-P (4-methyl-5-(β-hydroxyethyl) thiazole phosphate), *thiG* encoding for thiazole synthase, *tenI* encoding a tautomerase and *thi4* family genes which provides the thiamine thiazole synthase 2. Other than these, *thiD*, *thi5* and *thiC* are also involved in HET-P synthesis.

Thi1/thi4 in rice is responsible for transferring sulphur moiety from conserved cysteine residue (cys205) to a thiazole precursor. This step is necessary for HET-P biosynthesis by disulphide bond switching between adjacent cysteine resides, which eventually leads to TMP synthesis. On the other hand, thiC controls HMP-PP production which is also an integral part of thiamine biosynthetic pathway. Overexpression of these genes has also been reported during abiotic stress (Ribeiro et al. 2005). Overexpression of thiamine thiazoline synthase 2, which is a *thi4* family gene, along with *thiC* in rice led to increase in thiamine content in rice grains by \approximately fivefold. However, removal of peripheral layers of the grain during milling led to significant loss of thiamine. Other than these, so far, most attempts at thiamine fortification in rice have proved futile.

Some endosperm-specific promoters in rice have been identified, and promoter engineering has been carried out to overexpress *thiC, tpk* and *thi1*. The thiamine biosynthesis pathway in rice still shows a number of uncharacterized genes like *thiH*, *thiG/thiS*, *thiF*, *thiD*, etc. However, so far, effective modifications could only be made for *thi1 and thiC*, while proper characterization of these genes which might code for transporters, kinases, etc. may help in better targeting for thiamine fortification, so that more of it is available in edible parts.

Tissue-specific expression levels of thiamine can be checked by transcriptome analysis. Out of the rice expression databases like TENOR, PLEXdb, RiceXPro, etc., only RiceXPro provides data in the form of fragments per Kilobase of transcript per million mapping reads—also known as FPKM. However, even this database

does not provide a wholesome expression of thiamine biosynthesis enzymes. Transcriptome analysis with this database helped in studying expression of *ncs1, thiC* and three *tpk* genes. Most of these genes other than *tpk2* showed negligible expression in endosperm and whole seed. Synthesized thiamine and its precursors need to be properly localized in endosperm so that they are available as part of our diet. This requires overexpression of genes of thiamine biosynthesis pathway, as well as overexpression of transporters like Tpk, Put3 or Ncs1. This can be achieved by promoter engineering and introduction of cis-acting activators.

Tissue-specific expression studies must be done with variable thiamine levels to identify the key enzymes in biosynthesis pathway. Clustered Regularly Interspaced Short Palindromic Repeats-Cas9 (CRISPR-Cas9) technology also makes gene editing much simpler and easier. Thus, better characterization and overexpression of genes in biosynthetic pathway may lead to further betterment of this fortification attempt (Pathway Editing Targets for Thiamine Biofortification in Rice Grains published: 10 July 2018. https://doi.org/10.3389/fpls.2018.00975).

4.2 Mineral Fortification

4.2.1 Iron

Though iron deficiency is one of the leading micronutrient deficiencies in the world, very less work has been done to fortify crops with iron (Ahman et al. 2000; Datta and Bouis 2000; Goto et al. 1999; Kracht 1999; Lucca et al. 2001b; Yip 2002). One such attempt was being made to transform soybean *ferritin* gene in an indica rice line to fortify it with iron and zinc (Vasconcelos et al. 2003). Ferritin is a storage protein for iron, which stores it in non-toxic form and makes it available for metabolic functions at a slow rate (Briat 1996; Goto et al. 2000; Murray-Kolb et al. 2002; Theil 1987). Most of the iron, which is stored in rice grains, is lost during the milling process. In this study conducted by Vasconcelos et al. (2003), the indica rice line IR68144-3B-2-2-3 was transformed with soybean *ferritin* gene, under the control of *glutelin* promoter and levels of iron in transgenic rice was studied. Biolistic approach was used to transform indica variety of rice with ferritin protein. The gene of interest from soybean *Glycine max L.* was introduced in pGPTV plasmid under the control of the endosperm-specific promoter *GluB-1*. It was co-transformed with a plasmid pGL2, which contains *hpt* as selectable marker. The regenerated plants were initially screened using PCR techniques, which was followed by assaying the gene activity in the transgenic line using Western blot and immunochemical methods (Christou et al. 1991; Datta et al. 1998; Murashige and Skoog 1962). The gene was stably inherited in a Mendelian fashion in transgenic lines (as seen by the Southern blot analysis of transformed against non-transformed plants). Immunological methods confirmed that ferritin protein was localized in the endosperm region of rice grain, giving it a dark brown coloration (Vasconcelos et al. 1991). A 4.4-fold increase in iron content in transgenic rice was observed in this attempt, where they obtained as much as 71 μg/g iron per gram of unpolished rice. Observations also indicated a high content, up to 31 μg of iron even in polished seeds of many transgenic lines. Consuming such

transgenic rice varieties, as much as 33% (considering consumption of 300 g rice per day) of the RDA recommended iron intake can be met (Vasconcelos et al. 1991). The transgenic rice has been tested on rat models deficient in iron, which showed substantial replenishment in haematocrit values and haemoglobin concentration. It was shown by some studies that vitamin A increases the bioavailability of iron (Murray-Kolb et al. 2002). Vitamin A deficiency can also be correlated with prevalence of anaemia in both human and rats. Attempts are presently being made to create doubly transformed variety of rice, by introducing β-carotene pathway in this transformed indica rice variety. This may meet iron, zinc as well as vitamin A requirement and increase iron bioavailability in the fortified crop (Hinderaker et al. 2002; Semba and Bloem 2002).

4.2.2 Zinc

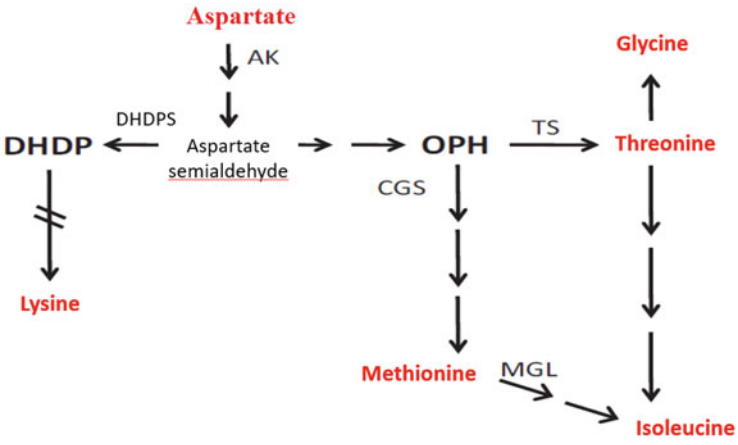
Zinc is another important micronutrient, whose deficiency can be associated with problems in pregnancy, retarded growth, genetic abnormalities and even impaired immune and neuronal development (Allen 2002). This deficiency can also be addressed by transforming local varieties of disease resistant rice with *ferritin* gene. The exact pathway of ferritin action in overcoming zinc deficiency is unknown. However, it has been clearly observed that the storage of iron and zinc are correlated, and increase in levels of one influences the other in similar pattern. Phytosiderophores have been shown to mobilize both iron and zinc (Black 2001). This hints at a common regulatory process to sequester iron and zinc. Consistent with this thought, transformation efforts also show that rice lines having higher iron content also show a higher zinc content. Transformation of the same indica line of rice IR68144-3B-2-2-3, with *ferritin* gene led to increase in levels of zinc when compared with the control. While the control showed mean zinc content of 33.6 mg/g, ferritin containing transgenic rice had zinc contents ranging from 34.9 to 55.5 mg/g. Thus the pooled mean values showed significant increase in zinc contents in the endosperm of transgenic rice (Vasconcelos et al. 1991). For fortification to be beneficial, it should withstand milling and polishing of rice. Comparing non-transgenic control with IR68144 transgenic lines, it was found that zinc content in transgenic rice remained higher even after milling and polishing when compared with the non-transgenic counterpart. The level of zinc in these transgenic varieties may vary according to the choice of initial cell line, yet variations are moderate (Goto et al. 1999; Lucca et al. 2001b). For the higher iron/zinc content to be beneficial, bioavailability of the same should be studied. However, as ferritin is a natural source of iron and zinc in plant and animal world during special stages of development, its bioavailability is not a problem. The levels of iron and zinc in milled grains are found to be quite diverse (depending on differences in processing). This makes it difficult to quantify the exact amount of micronutrient supplied, which could help deduce the consumption needed to meet the RDA for the same (Gregorio et al. 2000). The soil composition and water conditions can also affect the mineral availability in a particular crop. In close association with iron fortification, zinc fortification as well can help meet the regular dietary requirements of these micronutrients (Gregorio et al. 2000).

4.3 Amino Acid Fortification

Humans are monogastric and thus cannot synthesize all essential amino acids. In developing countries, maximum population depends on cereal crops, which leads to deficiencies in essential amino acids, especially Lys and Met (De Clercq et al. 1990; Hoffman et al. 1988). Such deficiencies can lead to reduction in disease resistance, lower blood protein levels and physical and mental retardation in kids. Due to this, 30% of the population [World Health Organization (WHO) estimation] in developing countries suffer from Protein-Energy Malnutrition (PEM). In the 1990s, attempts were made by to genetically modify seed storage proteins with additional codons for lysine and methionine (Galili and Amir 2013). In rice seeds, one such chimeric gene derived from rice glutelin and lysine rich winged bean protein showed about 45% increase in Lys levels (Beauregard and Hefford 2006). Similar attempts were also made for increasing methionine levels in storage proteins. Two of such successful attempts are described in the next sections.

4.3.1 Lysine

In most of the developing countries, lysine and methionine insufficiency in diet is a common problem as the poorer population cannot supplement their diet with sufficient amount of protein sources like meat, milk, egg or other protein-rich plant sources (Galili and Amir 2013). These two amino acids belong to the aspartate family pathway, which is also responsible for the synthesis of threonine and isoleucine. Lack of these two essential amino acids in diet reduces the nutrient content of the cereal grain to 50–75%. This may lead to reduced disease resistance, blood pressure reduction, retarded mental and physical development, etc. These syndromes are known as PEM or protein and energy malnutrition. According to statistical estimates made by WHO, 30% of population in developing countries are suffering from such malnutrition (Galili and Amir 2013).



The above figure shows the aspartate family pathways that leads to the formation of amino acids, lysine, methionine, threonine and isoleucine marked in red (Galili and Amir 2013). Here the abbreviations denoted are as follows: AK aspartate kinase, CGS cystathionine c-synthase, TS threonine synthase, DHDPS dihydrodipicolinate synthase, MGL methionine c-lyase.

The enzymes shown in the above pathway are lucrative targets for genetic modification which may improve the Lys-Met content of rice. Yet, there are some limitations of this approach. Significant increase in lysine and methionine contents are seen, but the localization is mainly in vegetative parts of the plant rather than the seed (Bright et al. 1982). Moreover, enhancement of these amino acids may lead to deleterious effects on plant growth. Localization problem may be addressed by using seed storage-specific promoters. In case of rice, *glutelin* promoter leads to high expression of endosperm-specific proteins. Recombinant endospermic proteins may also be developed which are rich in lysine and methionine. The success of these methods however depends on expression studies. They must also be stably inherited and should have least deleterious effects on plant (Kawakatsu et al. 2010).

There can be two ways of improving amino acid and protein contents of cereal grains by genetic engineering: (i) transform with amino acid rich proteins, so that the overall protein content increases, and (ii) targeting metabolism and increasing specific amino acid in free form (Fickler 1995). Seed storage proteins are localized within the endosperm. In rice, genes encoding seed storage proteins with additional codons for aspartate family-derived amino acids (lys and met) could be introduced. Most of these storage proteins were however highly unstable due to the modifications made (De Clercq et al. 1990; Hoffman et al. 1988). Some successes have been reported where the expression of a chimeric gene for lys-rich protein from winged bean was expressed under rice *glutelin* promoter. Lys levels in this rice variety were reported to be more than 45% higher when compared with non-transgenic lines (Wenefrida et al. 2009).

In cereal grains, lysine and tryptophan are the two most limiting amino acids. In seeds, storage proteins, known as SSPs, account for about 70% proteins in cereals. Binding protein BiP, an endoplasmic reticulum (ER) stress response protein, upon overaccumulation in the endosperm leads to intense downregulation of SSPs. This in turn leads to an increase in non-SSP protein load to maintain the balance which increases the free lysine content (Kawakatsu et al. 2010). BiP has a molecular weight of 73,000 with 62 lysine residues in its molecule. Lysine content in BiP is 9.4%, which is higher than most seed storage proteins. Reduction in 13 KDa prolamins (an SSP) led to increase in total lysine content of rice grains, as lysine-rich glutelins and BiP were seen to increase. Thus, BiP overexpression in endosperm-specific manner led to the following observations in transgenic rice: (i) rice grain weight was reduced by 48% (due to decrease in SSPs), and (ii) total lysine content was increased by a massive 2.9 fold (Kawakatsu et al. 2010). Hence, it could be inferred that BiP overaccumulation led to the increase in total lysine content accounting up to 10 μmol/g dry weight of seeds (other amino acids did not show much marked change in concentration). This corresponds to 27% of the total increase in lysine content, which shows BiP itself is one of the major factors controlling rice lys

content (Kawakatsu et al. 2010). Prolamins (an SSP) deposit in non-digestible prolamin bodies-I (PB-I) and cannot be efficiently absorbed as nutrients. On the other hand, BiP, glutelin, etc. which were increased via transgenic approach are completely digestible by pancreatic digestive juices which helps in their proper accumulation in humans (Kawakatsu et al. 2010). Thus, the BiP overexpressed transgenic rice could be currently used for animal feed or supplemental feed. Crossing this trait with a higher yielding rice cultivar would improve commercial production, and the thought of passing all regulatory measures to make it fit for humans is not only attractive but also essential.

4.3.2 Methionine and Cysteine

Methionine is one of the essential amino acids which cannot be produced by our body, but needs to be supplemented through our diet. Rice is low in sulphur-containing amino acids, having only 0.18% of Met and about 0.11% of Cys (Fickler 1995). Methionine can be further converted to cysteine irreversibly, thus making it the only essential source of sulphur in the body. Sesame seeds have high methionine content. About 30 years ago, United Nations declared Sesame flour as an important supplement for methionine deficiencies. Later it was deduced that 2S albumin in sesame is the main protein supplying this sulphur rich amino acid (Tai et al. 1991). Sesame 2S albumin is formed as a 17 kDa precursor protein with a signal peptide that directs it to endoplasmic reticulum (ER). It undergoes cleavage and disulphide bridge formation to give the final S2SA with 4 kDa and 9 kDa subunits linked by disulphide bonds (Hasegawa et al. 1978). Attempt was made to fortify rice with methionine and cysteine by introducing S2SA polypeptide cDNA under the control of rice *glutelin* promoter (which is strongly expressed in all parts of rice, especially in aleurone and sub-aleurone layers), using *Agrobacterium tumefaciens*-mediated transformation (Hasegawa et al. 1978). This chimeric gene product pGlu2S was introduced in japonica cultivar TNG67 of rice, and the transgenic plants were studied. Ten transgenic lines were created by the insertion of pGlu2S in the genome. The stability of the protein S2SA was important for nutritional enrichment. S2SA synthesis was found to occur in matured rice grains. To analyse the localization of the S2SA protein, milled rice and bran were separately subjected to Western blot analysis. This showed that 90% of S2SA accumulated in rice endosperm and was present in milled rice, whereas only 10% was expressed in bran. The rice harvested from the transgenic lines also appeared to be less transparent compared to the control. Crude protein content in transgenic rice was found to increase by 0.64–3.54% when compared with non-transgenic lines. While the wild type showed a 0.21% methionine content, transgenic lines expressed methionine in the range of 0.27–0.37%, which is considerably higher than wild type. In five transgenic lines out of ten, the methionine content was increased by 28–76%.

In some cases, where there is substantial increase in expression of sulphur-rich protein in transgenic lines, it does not ensure increased cysteine and methionine levels. These proteins may be synthesized in place of other proteins, or depriving other proteins of sulphur containing-amino acids, keeping the overall sulphur contents same. Fortunately, that was not the case observed in S2SA transgenic

lines, which showed 76% and 68% increase in methionine and cysteine levels. Sesame seed proteins do not have reported allergenic effects. There is no visible difference in appearance, yield or growth rate between the transgenic and non-transgenic crops. It is being considered that this current transgenic line developed with sesame S2SA will be appropriate for human and animal consumption. It is expected that a large-scale field trial and testing with animal models will soon follow to make proper use of this technology.

4.4 Soybean Glycinin

In soybean, glycinin is the main protein forming 65–80% of the protein fraction and about 30% of the seed mass. Glycinin can lower serum cholesterol levels and blood pressure (Sugao et al. 1990); it also serves as a good source of lysine and other amino acids which created urge among researchers to attempt fortification of rice with soybean glycinin. Rice cultivar Matsuamamii was transformed with the glycinin gene *AlaBlb* from soybean under the control of rice *glutelin* promoter, using biolistic approach. The transgenic rice produced using this method was visually indistinguishable from non-transgenic variety in terms of weight and appearance and showed high nutritional properties (Momma et al. 1999). The transgenic rice so obtained was thoroughly tested in Japan Food Research Laboratories in Osaka, Japan. These experiments included determining the moisture, lipid, fibre, ash, carbohydrate, vitamins and protein contents of powdered milled rice. Protein was quantified by determining the total nitrogen content using Kjeldahl method. Amino acid content was analysed after hydrolysis with HCl in vacuum, followed by neutralization and quantification using automated amino acid analyser (Keiko et al. 1999). The levels of protein in the transformed rice were significantly more (about 20%) compared to the wild type, with an additional advantage of low moisture content. The amino acid that showed significant increase in level was lysine (from 0.25 g/100 g to 0.3 g/100 g) and phenylalanine (from 0.32 g/100 g to 0.41 g/100 g), along with marked increase in almost all amino acid contents (Keiko et al. 1999). No significant changes in quantities of lipid, fibre, ash and carbohydrates were observed in the transgenic rice when compared with the control. Other than proteins, fatty acids like palmitic acid, linoleic acid and omega acids also showed increased levels. There was appreciable increase in vitamin B_6 levels (up to 50%) (Keiko et al. 1999).

Simulation of human digestion was used to study the digestibility of expressed glycinin. For this, SGF (which simulated the gastric juice) and SIF (which simulated the intestinal juice) were used. It took 10 min in SGF and 30 min in SIF to completely digest glycinin protein. Some proteins of size 50 kDa were seen after SIF digestion; however, they were characterized to be the inherently indigestible proteins present in rice.

Transgenic rice expressing soybean glycinin protein is highly nutritive, physiologically noteworthy and easy to process and digest (Keiko et al. 1999). During the development of this transgenic variety, care was taken to make it socially more acceptable. The crops generated using novel biotechnological approaches should be

at least as safe as the traditional counterparts. This gave the concept of "substantial equivalence" (Keiko et al. 1999). For sustainable equivalence, there must be a wholesome identity between the transgenic and non-transgenic crop, in terms of all characters except for the one under manipulation. If the crop so produced deviated from the traditional crops in varied aspects, then it should undergo strict checking to ensure its safe consumption. In this transgenic rice, parameters such as protein, vitamin B_6, and some fatty acid contents varied slightly. Following the concept of 'substantial equivalence', attempts were made to make the crop meet the safety and anti-allergenic standards. Protein level enhancement of 1.2 times was desirable. Vitamin B_6 and fatty acid content changes in transgenic rice were also seen not to affect human health as they were within normal levels (Keiko et al. 1999). The reason for such changes of protein, fatty acid and vitamin contents could be narrowed down to position effect, where introduction of new genes affects the expression of the genes already present, and metabolic interference, where the new gene products interfered with metabolic pathways of the traditional crops. Such metabolic interferences by heterologous gene expression cannot be disregarded, as it could lead to production and accumulation of hazardous compounds. Thus, this transgenic crop is currently being tested for any undesirable or unexpected changes due to disruption of metabolic pathways, before it can be introduced in the market (Shirai et al. 1998).

5 Conclusion: Trying Out Fortified Rice

Attempts have been made to try out fortified rice since the 1970s. From January 1971 to July 1975, a trial of fortified rice was attempted in three sets in 29 villages of Chiang Mai, Thailand. Children in these villages suffered from malnutrition as compared to the middle class Thai children. One set of fortified rice contained lysine, threonine, thiamine, riboflavin, vitamin A and iron. Second set was fortified with the vitamins and iron, but no amino acids, while the third set was placebo. However, the study was faltered as hardly 10% of the rice consumed was fortified. The children did not meet daily caloric values due to low calorie density food and lack of palatability of their diets (Gershoff et al. 1977). A 6-month trial in Philippines encouraged the use of iron fortified rice among 218 school students. The results were in tandem with expectations and showed higher blood haemoglobin levels and less occurrence of anaemia among these children (Florentino 2001). Similar studies were also conducted in India, Brazil and Mexico which showed positive effects of iron-fortified rice (Angeles-Agdeppa et al. 2008; Beinner et al. 2010; Hotz et al. 2008; Moretti et al. 2006). This gives us a hope that when consumed in adequate amounts, fortified ice could supply required amounts of micronutrients and prevent deficiency diseases. Following proper regulatory measures, commercialization of such transgenic rice should be encouraged in order to make best use of them in eradicating malnutrition problems.

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Golden Rice: Genetic Engineering, Promises, Present Status and Future Prospects

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Abstract

Since essential micronutrients are not produced within humans, it has to be taken via the food. Genetic engineering provides one such solution to enhance the quality of our food. Since rice is one of the primary staple food across the world, a new variety of rice called Golden Rice is developed as a result of genetic engineering to produce beta-carotene (provitamin-A), which is a source of vitamin-A. The endosperm of rice lacks the provitamin-A, and hence among the rice consuming population, it causes vitamin A deficiency. Therefore to overcome this threat, biofortification method has been used which involves deliberately enriching the endosperm of rice with provitamin-A carotenoids. Golden Rice was developed to distribute it to the farmers of the poor and underprivileged of the developing countries without any restrictions or any

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charge. It targets a vast population of women and children with or at the risk of vitamin A deficiency in many developing countries of Asia and Africa. The endosperm tissue of the immature rice was examined by a variety of biochemical assays, and it was observed that geranyl, geranyl-pyrophosphate (GGPP) is present and it is the initial precursor that is converted into the alpha- and beta-carotene. However, the GGPP is not further metabolized, and there is transcriptional inactivity of specific genes involved in the production of isoprenoid compounds. Hence transgenic approach was used for the synthesis of beta-carotene through manipulating *Psy* (Phytoene synthase) and *crtI* (carotene desaturase) genes. As per the food regulations of genetically modified crops; genetic engineering of food is considered way better than the conventional crop breeding technologies. It provides for modifications subjected to the genetic make-up of the crops thus aiming to enhance the nutrition, production, medicinal traits and resistance (to diseases) of the crops.

Keywords

Beta-carotene · Biofortification · Genetic engineering · Golden Rice · Vitamin A deficiency

1 Introduction

Micronutrients constitute an essential part of a healthy lifestyle but are not produced within humans. Thus, micronutrients are taken up through the food. The primary source of food is the staple crops but often lack one or more micronutrient and hence needs to be complemented by other foods. Due to the complex genome of some crop plants, lack of genetic variability of the desired traits and issues regarding sterility, conventional breeding is not always regarded as a viable option. For such cases, genetic modification is the sole viable option. Due to the identification of genes associated with interrelated biochemical pathways, some tools have been developed to yield few micronutrients in the staple crops. Thanks to the development of sophisticated techniques over the past years, the knowledge of the biosynthetic pathways have been growing at a rapid pace. It became possible because all the organisms are related due to the sharing of an adequate number of genes and the metabolic functions, allowing us to attain conclusions from one to another species. It also allows us to research model organisms possessing shorter life spans at the same time benefitting from the extensive genetic material available like the gene expression data and of course from the sequenced genomes (Della Penna 1999; Grusak and Della Penna 1999). The explanation of metabolic pathways and their interrelations within the plants remains a big challenge mostly cause of the numbers. Plants pose as very efficient chemical factories. A single leaf of *Arabidopsis* possesses not less than 2000 distinct substances, while the whole plant kingdom can form 200,000 or more of distinct chemicals (Pichersky and Gang 2000). Most of these substances are necessary for the adaptation of the plant to specific environmental conditions and

are called secondary metabolites. While few of these substances fulfil as essential nutritional substances to humans and are called vitamins in its organic form and trace elements in its inorganic form. Together they are called micronutrients.

Vitamin A depicts a group of C20 carotenoid derivatives such as retinol and its esters, retinal and retinoic acid. Within humans, the provitamin A carotenoids are cleaved off from the middle of the molecule to form vitamin A retinoids. It is commonly found in animal-derived food such as eggs, fish products and liver. Plants too help in supplying vitamin A to humans by producing provitamin A carotenoids such as b-carotene. The economic wealth of a region or country defines the amount of vitamin A which can be derived from a plant source. For example, only 26–34% of vitamin A is utilized by Americans in the form of provitamin A carotenoid (http://ods.od.nih.gov/factsheets/cc/vita.html). While in several developing countries such as the rural parts of Bangladesh, provitamin A carotenoids play a significant role in providing vitamin A, where the calorie intake is only 3% from animal sources but approximately 80% from rice (Bouis 2000). Vitamin A plays a vital role in providing vision, epithelial cell repair and growth, immunity, reproduction, bone growth, maintenance of intestinal and urinary tract along with linings of eyes and epithelium of the respiratory tract. It is also essential for adult gene regulation and embryonic development. As a result of its deficiency, there is an increase in the susceptibility of diseases and several disorders. Night blindness is the first symptom that occurs due to VAD. VAD over a long period leads to dryness of the conjunctiva which is further slowly extended to the cornea of the eye (xerophthalmia). The dryness results in the shrivelling of the cornea which then becomes ulcerated leading to a condition known as keratomalacia. It is lastly followed by enhanced inflammation and infection occurring within the eye leading to irreversible and complete blindness.

Along with the occurrence of eye diseases, VAD leads to an increased susceptibility to diarrhoea and measles among children thus resulting in enhanced child mortality rate. Such an increase in the infectious condition and mortality are evident before the emergence of xerophthalmia. Various subclinical defects such as reduced immunity, impaired mobilization of iron and disrupted cellular differentiation are also recorded in response to VAD (Sommer and Davidson 2002).

The vastness of the problem associated with this malnutrition can be governed from various studies carried out by the World Health Organisation (WHO) which shows decreased child mortality by ~23% if VAD is corrected (Beaton et al. 1993). According to WHO statistics, VAD poses as a public health problem in over 118 countries across the world and is touted to be affecting approximately 140–250 million preschool children. Each year, an estimated 250,000–500,000 vitamin A-deficient children are blinded, of which 50% of these children die within a year of losing their eyesight (Bhutta et al. 2013; Howart 2000). A prime cause of blindness during childhood across the world is attributed to VAD. It is perceived to be a leading contributing factor in an estimated 1–3 million children dying each year, mainly due to the complications arising because of malnutrition which can be decreased by providing better nutrition to the children, including vitamin A.

2 Why Golden Rice?

During the 1990s, Potrykus and Beyer from Zurich as well as Freiburg developed Golden Rice as a result of a call by Rockefeller Foundation to curb vitamin A deficiency using an efficient solution implying plant breeding. It led to the development of a first novel variety of rice containing 1.6 μg/g of carotenoids within the endosperm. When Syngenta was used as a partner of Golden Rice in the second generation, 37 μg/g of carotenoids was detected which proved to be sufficient in fulfilling half of the daily requirement of vitamin A present in 60 g of uncooked rice (Paine et al. 2005). Carotenoids, synthesized by de novo pathway within the plants, play principal physiological roles as precursors of signalling molecules, as photosynthetic pigments, as an essential constituent of a healthy diet, as the precursor of vitamin A and as antioxidants. VAD in developing countries poses as a public health problem which has led to promoting the efforts towards biofortifying the plant-based foods with b-carotene leading to the formation of 'Golden' crops (Ye et al. 2000; Beyer et al. 2002; Paine et al. 2005). After the development of Golden Rice, various biofortified crops have been developed by utilizing transgenic as well as conventional breeding methods. Studies regarding the bioavailability of vitamin A have determined the efficiency of various golden crops towards the maintenance of vitamin A status (Fig. 1).

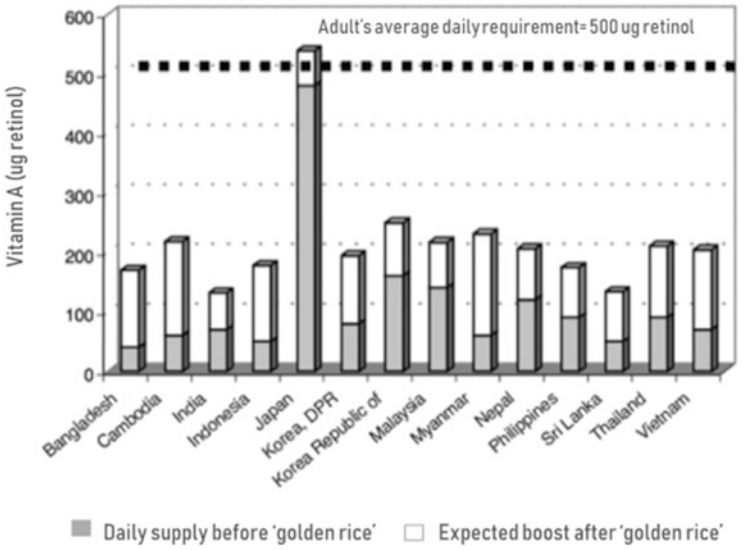

Fig. 1 Golden Rice solving vitamin A deficiency by supplying it in countries where rice is a staple food

3 Biofortification

In several countries, malnutrition of the micronutrients poses as a severe public health issue. For several decades, fortification and supplementation-based solutions have been utilized to curb this problem. But interventions such as these are economically unsustainable due to the need for on-going funding. Studying the vitamin A supplementation programmes showed a cease in its coverage in 103 priority countries over the past decade, revealing a high rate of year-to-year fluctuations (UNICEF 2007).

Biofortification refers to 'fortification' of agronomically primary crop plant tissues through their biochemical capacity. As soon as the crops are formed, the cost of this method forms only a small part of the cost of supplementation. The crops thus obtained are meant for distribution via the traditional agricultural pathways or through the local trade to reach the target people such as the urban and rural people, specifically those residing in remote regions. Golden Rice though requires a bit more than the average cost of reliable seed production systems for the continuous deployment of it after its introduction. The cost of production is the same as that of any other rice. The breeding cost is average with an approximate cost of $ 4 million per variety spread over a decade (Nestel et al. 2006). This cost of $ 4 million amounts to approximately 0.2% supplementation expenditure of global vitamin A in the same method mentioned above over a decade (Nestel et al. 2006). Development and regulatory approval for a new transgenic crop amount to 5–8 times higher, which represents a mere fraction of sustained costs required for classical public health solutions. As soon as a more precise regulatory requirement is established, these high costs may potentially be reduced. With these advantages and along with genetic engineering biofortified rice is formed to help curb diseases as a result of vitamin A deficiency, which is widely prevalent in tropical countries.

4 Intent and Role of Golden Rice

Golden Rice is a generic name of genetically modified rice which produces provitamin A (b-carotene) within the endosperm. The name is taken from the yellowish colour of the rice which is visible after it is milled and polished, a routine procedure done to get rid of the outer layers of the grain. Genetic engineering was employed to develop Golden Rice as the rice germplasm is incapable of forming carotenoids within the endosperm (Ye et al. 2000).

Due to the genetic transformation, the Golden Rice grains accumulate provitamin A, and this is expected to provide the essential micronutrient through agriculture sustainably to the target population. As soon as the prototype Golden Rice was produced, it has undergone vigorous research to enhance the content of provitamin A, to get the scientific basis established for its carotenoid complement as well as comply effectively with the regulatory requirements. At present, the main focus is on the novel avenue for public sector research that is in providing the grain in farmer's hands which is taken forward with the help of international research

consortia. Further research will be carried out on Golden Rice to enhance its nutritional value.

5 Golden Rice Development

Peter Beyer from the Freiburg University and Ingo Potrykus from the ETH institute in Zürich have worked on the development of rice that is responsible for the production of provitamin A in the endosperm. Golden Rice was produced by adding two genes to the genome of rice one from *Erwinia uredovora* which is a soil bacterium which codes for *crtI* (carotene desaturase) gene and another from daffodil (*Narcissus pseudonarcissus*) which codes for gene *psy* (phytoene synthase). These two beta-carotene synthesis genes enable the production of provitamin A in rice grain (Burkhardt et al. 1997; Ye et al. 2000; Paine et al. 2005). Since the production of provitamin A is a multi-step process, so initially it was thought that gene *lcy* (lycopene cyclase) is also involved in the production, but through further research, it is now confirmed that it is already synthesized in the endosperm of wild type rice.

The metabolic engineering related to the production of Golden Rice is quite simple and based on the fact that the wild-type rice plant possesses all genes responsible for the synthesis of beta-carotene and while this system is active in the leaves but switch off in case of the endosperm. Hence two genes, namely, *crtI* gene and *psy* gene, were added to the genome of a rice, and it was located under the regulator which is endosperm-specific promoter so that they are only expressed in rice endosperm. The *lcy* gene is exogenous, and it has a peptide sequence attached to it which helps it for transportation, and thereby it is targeted into the plasmid where it led to the formation of compound geranylgeranyl diphosphate. The gene obtained from bacteria *crtI* is significant to complete the pathway, subsequently, to the lycopene synthesis, it catalyses several steps in the carotenoids synthesis, whereas in plants these steps involve more than one enzyme (Hirschberg 2001). The end product formed in the pathway is lycopene, and hence plant becomes red if it accumulates lycopene. Based on current analyses, it has been observed that plant contain many endogenous enzymes which further convert the lycopene formed in endosperm to beta-carotene and hence giving the rice unique yellow colour for which it is named (Schaub et al. 2005). *SGR1* is known as Golden Rice which produces approximately 1.6 μg/g of carotenoids.

In the initially produced Golden Rice, the concentration of provitamin-A was deficient, and hence it was not very useful. Subsequently, people have to take it in higher amounts to reach up to the recommended level. Several years later an improved version came in which instead of *Narcissus* maize gene was used and this new Golden Rice results in production of 23 times more provitamin-A when compared with the initial Golden Rice and hence it was proved to be more useful to combat against the Vitamin-A related deficiency (Paine et al. 2005).

6 Biosynthesis Pathway (Fig. 2)

Genetically modified (GM) rice was further tested, and various nutritional experiments were conducted in China involving children between the ages of 6–8 years (Tang et al. 2012). Data was collected and found that this GM Golden Rice provides as much vitamin A as produced by the provitamin A capsules which could be even higher than spinach. This research calculated that approximately 60% of vitamin A is produced in children by consuming about 100–150 grams of boiled Golden Rice. Moreover, among the rice-eating populations, Golden Rice provides sufficient vitamin A (IRRI 2013a).

Golden Rice is produced through the process of biofortification which is used to increase the amount of particular nutrient in the food crops such as vitamins, zinc and iron. Now with the help of this technique, it is possible to cultivate a particular crop with higher nutrient content, and thereby people can become self-sufficient. The process of biofortification can be attained through the method of conventional

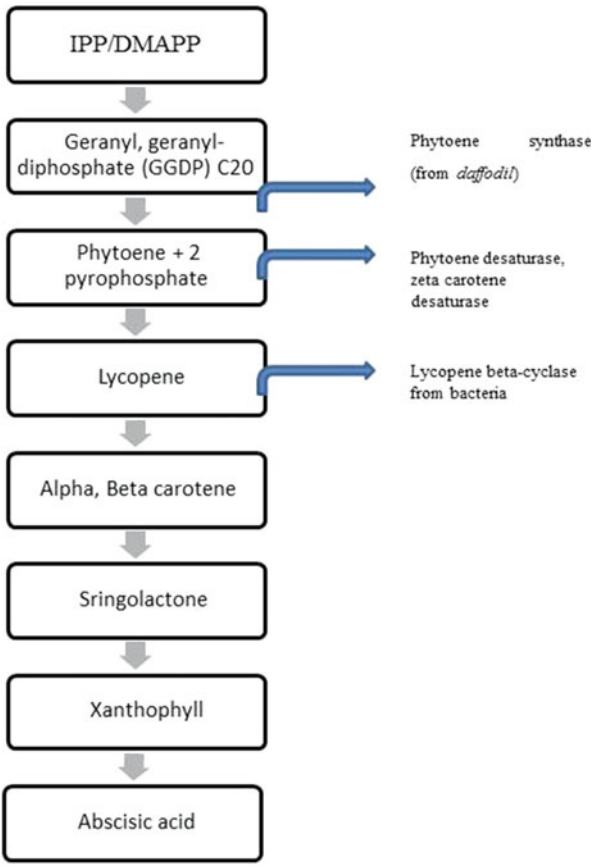

Fig. 2 Pathway overview: Biosynthesis begins with a small (C5) compound which is isopentenyl-diphosphate (IPP) along with its isomer DMAPP (dimethylally-diphosphate, due to chain elongation C20 compound which is geranylgeranyl diphosphate (GGDP) is formed. GGDP molecule acts as a precursor and two molecules of GGDP in head to tail fashion joins to form phytoene-A which is a first colourless carotene molecule. As a result of desaturation reactions lycopene formed which is coloured chromophore. Further cyclization reactions result in formation of alpha- and beta-carotene. Oxygenation reactions form xanthophylls, and various important biological molecules are formed from it like abscisic acid

breeding methods such as genetic modifications. For example, as in the case of Latin American beans, the amount of iron through breeding methods had increased from 50 to 90 milligram per kilogram (Petry et al. 2015; Haas et al. 2016).

Crossbreeding programmes are used to enhance the level of provitamin A content in *cassava* and maize, zinc in rice and iron in pearl millet (Lamberts and Delcour 2008; Saltzman et al. 2013). Like Golden Rice, similar work has been done by Australian and Ugandan scientist on banana variety to produce provitamin A to combat vitamin A deficiency.

Profs Ingo Potrykus and Peter Beyer gather information that were able to challenge this feat. Their development showed that for turning out the Golden Rice into a reality, they require only two transgenes (Ye et al. 2000). The first transgene (*PSY*) uses the geranylgeranyl diphosphate synthesized endogenously and forms phytotene which is a carotene without colour and has triene chromophore (Burkhardt et al. 1997). Another gene is *CRTI* bacterial carotene which adds four double bonds and thus introduces conjugation. During 1993–1999 combined work has been done by prof Peter Bramley from Royal Holloway College, UK, and Peter Beyer in which they work on genetically modified tomatoes and they found that the advantage of utilizing only bacterial *CRTI* phytotene desaturase gene instead of using many plant desaturases (Romer et al. 2000).

Further down the enzymes such as alpha- and β-carotene hydroxylases (*HYDs*) and lycopene cyclases (*LCYs*) are synthesized in endosperm of natural wild type rice grain. Whereas, enzymes such as *PSY* and also carotene cis-trans isomerases (Chen et al. 2010) vizcis-trans isomerase (Isaacson et al. 2002; Park et al. 2002; Yu et al. 2011) plant carotene desaturase ζ-carotene desaturase, phytoene desaturase (*PDS*) are not produced. In the transgenic plant, *CTR1* and *PSY* help in the production of lycopene which acts as a substrate for other enzymes present downstream the pathway and subsequently allows the formation of the products.

The explanation is based on the fact that *PSY* transgene is enough for the production and accumulation of phytoene, but it does not produce the products which are desaturated (Burkhardt et al. 1997), and it is due to the absence of desaturase in its active form such as *PDS*. Likewise, if *CRT1* is expressed alone, then it will not result in the production of the coloured product in the endosperm of rice since it lacks the activity of *PSY* transgene. The importance of *CRT1* lies in the fact that it can alone catalyse the reaction from phytoene to lycopene, whereas in plants two cis-trans isomerases and two desaturases are required for the same reaction. Moreover, hence the number of transgene for the above-said reaction is reduced to two.

The requirement of transgene *CRT1* conflicts with the occurrence of *ZDS* and *PDS* transcript in the endosperm of a wild-type rice grain, as predicted by the RT-PCR quantitative analyses (Burkhardt et al. 1997). This could be explained by the fact that it is present in a little amount of enzyme as compared to its mRNA. The endosperm of rice provides the complex requirement for the plant desaturase activity, and this is evident by the fact that rice endosperm results in the formation of coloured carotenoid by tissue-specific *PDS/ZDS* expression. The transgenic

approach was used for this investigation and not in vitro method due to the low-level expression of *ZDS* and *PDS*.

The primary sequence of *CRT1* was compared to the plant desaturase and was found to be unrelated. Also, the structure and mechanism of the reaction were investigated (Schaub et al. 2012). *CRT1* transgene structure when resolved was found to be more straightforward as compared to the plant-type desaturases, and data suggest that molecular oxygen is used as an electron acceptor in *CTR1*, whereas plants employ plastoquinone molecule as an electron acceptor and hence require complex redox reaction pathway (Beyer et al. 1991; Mayer et al. 1990; Nievelstein et al. 1995).

Through the mutants mutation in the plant *Arabidopsis*, it was found that the electron path is somewhat linked to the molecular oxygen produced via oxidase (Kuntz 2004). In the case of non-green carotenoid tissue, this complex redox pathway plays a vital role, whereas in plants, photosynthetic electron transport is thought to play a similar role in the chloroplast. Moreover, plants form poly cis-trans isomerases but do not form *CRT1* (Bartley et al. 1999), and hence cis-trans isomerase are not required. The *CRT1* transgene in its one single step is responsible for the formation of four double bonds.

Further, the activity of enzyme lycopene cyclase depends on the expression of particular rice gene in the endosperm like the formation of xanthophyll which is catalysed by the enzyme β-carotene hydroxylases (Tian and Della Penna 2004). Since lycopene does not accumulate in the endosperm of rice, its activity is not rate limiting. Due to the presence of intrinsic rice cyclase, Golden Rice is yellow.

Usually, in multi-step biosynthetic pathways, the flux of the pathway is controlled by the rate-limiting step which is overcome with increasing the level of the enzyme that is rate limiting and also by using one enzyme that is more active than others. In the case of *PSY*, it was recognized and not in case of *CRT1* (Al-Babili et al. 2006). Second generation Golden Rice *GR2* is capable of producing about 37 μg/g of carotenoids, of which 31 μg/g was found to be beta-carotene when compared to the first generation which produces 1.6 βg/g (Al-Babili and Beyer 2005; Beyer et al. 2002).

A schematic representation of the production of Golden Rice is given below showing four different steps. The enzyme which catalyses the steps are phytoene synthase which is not present in regular white rice but produced in genetically modified type, phytoene desaturase, zeta carotene desaturase and lycopene cyclase. *Erwinia uredovora* can catalyse step second and third (Ye et al. 2000). Initially, the lycopene cyclase from *narcissus* was also transferred into rice in the first version of Golden Rice which soon in the second version of rice became evident that it is already produced naturally in wild type rice grain (Beyer et al. 2002; Paine et al. 2005). The second version which is the improved version developed in 2005 possesses the phytoene synthase gene of maize rather than desaturase of *Erwinia* (Fig. 3).

Pathway steps	Wild type of rice grain	Golden Rice version 1	Golden Rice version 2
Step 1 Phytoene synthase	This is not found in the white type of rice	Phytoene synthase from *Narcissus* used	Phytoene synthase from Maize used
Step 2 Phytoene desaturase	Not found in white type rice grain	Bacterial desaturase with dual function	Bacterial desaturase with dual function
Step 3 Zeta-carotene desaturase	Not found in white rice grain	Bacterial desaturase both functions lycopene cyclase of narcissus phytoene synthase of *narcissus* phytoene synthase of maize	Bacterial desaturase
Step 4 Lycopene cyclase	Not found in white rice grain	Lycopene cyclase of *Narcissus*	Not found

Fig. 3 Synthesis of Golden Rice involves four steps. This figure shows that phytoene synthase in variety 1 is obtained from the *Narcissus*, and in Golden Rice variety 2, it is obtained from Maize. Also lycopene cyclase is found in Golden Rice variety 1, and it is not found in Golden Rice variety 2

7 Promises and Current Status

Javanica, indica, and *japonica* cultivars have been treated with Golden Rice events, yet their different biochemical aspects have not been studied. In Asia, the marker-assisted selection is used for introgression, which gives sufficient information for making this distinction. Normally, in order to introgress the Golden Rice trait within the rice cultivars, cross-breeding technique is necessary, mainly for *indica* and is adapted locally in regions with vitamin A deficiency.

The presently developed Golden Rice technology (*GR2*) utilizes two provitamin A pathway genes (Al-Babili and Beyer 2005). One out of the two genes codes for phytoene synthase enzyme, which is the major rate-limiting step in several plants in the production of carotenoid. The significant improvement in *GR2* technology over *GR1* involves the exchange of phytoene synthase from that of daffodil with maize, hence enhancing the formation of provitamin A (Schaub et al. 2005). While the other gene, *Crt1* derived from bacterium *Pantoea ananatis* (earlier *Erwinia uredovora)* codes for carotene desaturase, *Crt1* has been used as the plants require four genes (two for carotene cis-trans isomerases and two for carotene desaturases) in order to carry out the same sequence of reactions which leads to the formation of provitamin A precursor, lycopene. Thus, one transgene from a bacterium can serve for four transgenes of the plant.

The upstream enzymatic activities of phytoene and downstream of that of lycopene in the carotenoid synthesis pathway are all expressed actively within the endosperm of wild-type rice. Therefore, there is only a need for bridging the gap for reconstituting the whole sequence of carotenoid biosynthesis (Schaub et al. 2005). Over the past years, the trait has been introduced into few locally adapted varieties of rice from the original rice cultivar. It was carried out by a combined partnership of institutions from Vietnam, Philippines and India. Another staple food in more than 50 countries is plantain, the cooking banana. For example, in Uganda, its consumption reached to approximately 220 kg/person/year, though banana when eaten consists of only lower levels of minerals and vitamins. Since bananas comprise sterile triploids, it is hence propagated vegetatively as it is very difficult to propagate it through conventional breeding. Gros Michel, a variety of banana, was almost wholly lost to the 'Panama disease,' whose causative agent was fungus *Fusarium oxysporum*, as it was not possible to provide resistance to it via the breeding method. As the banana-based food lack iron, provitamin A and E, a project, led by the Queensland University of Technology in Australia, is underway for enhancing its nutritional value via genetic transformation (http://www.grandchallenges.org). Likewise, cassava and potato rank high among human staple foods. Moreover, they are being targeted for employing genetic engineering to enrich cassava and potato (Diretto et al. 2007) nutritionally.

The possible outcome of the genetically modified (GM) crops on nutrition, poverty and income of the developing countries continues to be a subject of controversy. Insights from India depicts that genetically modified crops are likely to provide employment and reduce poverty. Also, it favours farmers by increasing the crop yield to save insecticide money and gain more income. One such example is the Bt-cotton which is adopted by small-scale farmers in China, South Africa and India. Similarly, the potential impact of Golden Rice rich in b-carotene, an example of the second-generation technology, was predicted in advance. It shows that Golden Rice may significantly reduce problems associated with health which can reduce up to 40,000 child deaths every year. More public support, better technology delivery systems and efficiently working regulatory bodies are required to materialize such social benefits on a large scale to help the poor across the world.

The widespread utilization of Golden Rice is expected to elevate the situation in rice-eating regions. Although Golden Rice has not been introduced in the market, it is expected to be soon commercialized in a few Asian countries.

8 Nutrition and Health Benefits

Over-nutrition, poor choices of diet or malnutrition always hurt more than 50% of the population of the world throughout every person's lifetime. It is observed that the rich are less concerned about the food insecurity and are more concerned about having a healthy and nutritious diet and over-indulgence. Approximately 1 billion individuals around the world are affected by food insecurity. Every year an estimated 10 million children die due to malnutrition, with 178 million children being stunted

and another 150 million being underweight (FAO 2008, 2009; http://www.gainhealth.org/about-malnutrition/nutrition-facts). Mortality and morbidity due to over- and under-nutrition accounts as the tip of an iceberg given the global diet which is falling short in meeting the health needs of the world. As a result of this failure, there is insufficient physical and mental development of 100s of millions of kids, with reduced gross domestic products, loss of worker's economic productivity and an increase in global bill for the medical care. In such conditions, lack of food and nutrients affects the poor very severely, specifically of the under-developed nations (FAO 2008, 2009; http://www.gainhealth.org/about-malnutrition/nutrition-facts). Deficiency of iron, zinc, folic acid, iodine and vitamin A accounts for the world's leading micronutrient deficiencies, with almost half the population of the world suffering from one or more of such deficiencies (FAO 2001, http://www.gainhealth.org/about-malnutrition/nutrition-facts).

There is a reasonable possibility of *VAD* occurring along with other deficiencies such as that of energy, protein or other micronutrients with similar diagnosis (Graff 2009; http://www.gainhealth.org/about-malnutrition/nutrition-facts). Several international programmes have been developed that have reduced the problems associated with *VAD*, using modalities like supplement injections or pills, though such programmes are expensive thus not reaching a significant section of the people affected with it and also requires to undergo repeated treatments. As around 70% of the world's people suffering from *VAD* are rice consumers, with rice being the leading staple food among them, hence Golden Rice was developed as a complement to other programmes associated with improving the *VAD* (James 2009; http://www.goldenrice.org/Content3-Why/why1_vad.html).

At present, Golden Rice is still in the developing phase and undergoing safety evaluation. Once it gets approved by the food safety regulatory bodies, it shall be distributed along with information campaigns (Potrykus 2001; Mayer and Potrykus 2011; Zeigler 2014). Nonetheless, there are mixed reviews in the context of the sustainability and beneficial effects of Golden Rice (Small 2014). Stein and Qaim (2006) have devised a methodology comprising of ex ante assessment of Golden Rice which focuses on the health and nutritional benefits along with the socio-economic benefits of Golden Rice. An empirical study was conducted in India using this methodology (Stein and Qaim 2008). India accounts as one of the largest consumers of rice and is hence chiefly targeted for the introduction of Golden Rice. Also, as *VAD* is widely prevalent in India, 35 million preschool children affected with VAD live in India off the 140 million (UN 2005) (Fig. 4).

Stein et al. determined the disease burden related to *VAD*-attributable fractions of these outcomes, generating a disability-adjusted life year (*DALY*) method. Number of *DALYs* lost depicts the annual morbidity and mortality in combination. Based on the currently available health statistics, the burden is calculated as the situation with no Golden Rice. The next step involved deriving the current b-carotene consumption from nationally acclaimed food consumption data and establishing the possible shift that might be seen in the intake distribution due to Golden Rice consumption after its introduction. This step involved making assumptions based on the experimental data obtained and the estimates by the expert related to the efficiency of the technology

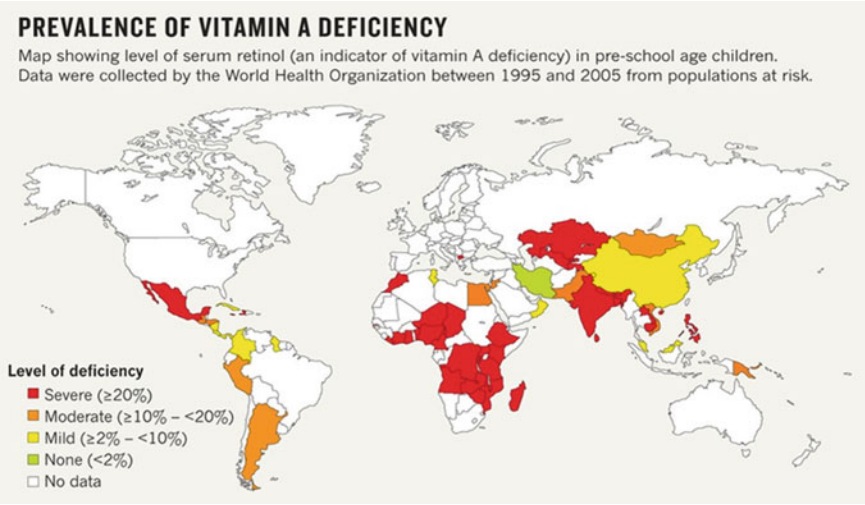

Fig. 4 Worldwide prevalence of vitamin A deficiency (VAD) from WHO data (1999–2005)

and its coverage in the future. The higher is the intake of b-carotene among the target populations the more improvement shall be observed in the individual's vitamin-A levels, hence decreasing the adverse health effects. The newly derived incidence rates were used to once again calculate the expected remaining burden, this time with the Golden Rice. The expected effect of this technology is depicted in terms of the number of *DALYs* saved, and it is determined as the difference in the disease burden without and with the Golden Rice (Stein and Qaim 2008). Based on the incidence numbers, *VAD* is responsible for taking the lives of more than 70,000 children in India, younger than 6 years of age. Going by these statistics, consumption of Golden Rice on a large scale can help decrease the disease burden by 59%, which amounts to giving life to approximately 40,000 every year.

The outcomes are most positive within poorest of the income groups, as the negative effect of *VAD* is related to the income of the target populations. Although Golden Rice itself is insufficient to eliminate the challenges faced due to *VAD*, the expected positive outcomes in public nutrition and health in itself are a considerable achievement. Similar results are anticipated in other rice-consuming countries affected with problems related to *VAD*.

Along with reducing the sufferings among the people and decreasing the health costs, the nutritional enhancements are also positively affecting the labour productivity of the populations. Macroeconomic model was used to reproduce the positive impact of Golden Rice across the globe (Anderson et al. 2005). The model accounted the health effects and the nutrition of the consumers as an elevation in productivity of unskilled labourers leading to estimated welfare gains of more than 15 billion US$ per annum across the world with most of the gains attributed to Asian countries. For instance, in China, the Golden Rice project is expected to lead an overall 2% growth in the national income (Anderson et al. 2005).

9 Cost-Effectiveness

The projected positive outcome of Golden Rice in combating problems associated with *VAD* is a cause of rejoicing for the future. Though from the economic point of view, it is necessary to analyse the cost of introducing such technology. The significant levels of investments for the production of Golden Rice on a large scale include research and its development, evaluation and distribution of the genetically modified rice to the target populations. Stein et al. analysed the Golden Rice technology in India by dividing these costs by the saved *DALYs* and then took into account the period when the benefits and the costs occur through discounting (Stein and Qaim 2008). This resulted in an optimal cost per *DALY* saved and is a standard measure of determining the cost-effectiveness of such public health interventions. By these calculations, the cost per *DALY* saved is coming out to be almost 3 US$ through Golden Rice. Another analysis based on the sensitivities approximated the amount per *DALY* saved to not more than 20 US$, which when compared to the standard benchmarks depicted its highly cost-effective approach since the World Bank categorize the cost-effectiveness of any health intervention if the cost does not exceed 200 US$.

10 Comparing Golden Rice with Conventional Vitamin A Intervention

In India, industrially fortifying the rice with vitamin A are scaling up its supplementation would be costing between 84 and 135 US$ per *DALY* saved. Such conventional interventions do not cost much for the production of the food fortificants or pills instead require a more significant amount of money for reaching out to the target populations within the remote areas and the regular monitoring. However, such costs are reduced with the introduction of Golden Rice. No doubt the initial cost of production of Golden Rice is slightly higher, but the recurring costs would be lower. This is possible as Golden Rice seeds are capable of spreading through the available informal and formal channels of distribution, and also the farmers can reproduce it quickly. Though there are problems related to its acceptance among the consumers, several efficient and appropriate strategies are required for convincing the farmers to take up Golden Rice in their fields. A very promising avenue involves combining the nutritional value of b-carotene with other important agronomic traits. Golden Rice is coming out to be a complementary *VAD* intervention due to its high cost-effectiveness. A single approach alone cannot tackle the issues related to *VAD* since all the possible interventions possess their weaknesses along with their strengths. In urban regions, industrial fortification and food supplementation might be excellently suitable, but Golden Rice is more likely to get adapted effectively in remote rural areas. In the longer run, immediate necessity of such interventions might decline due to a decrease in poverty and growth in the economy which shall be contributing to the diversification of the diet among the target populations.

With the invent of modern molecular plant breeding techniques which utilize recombinant DNA technology and DNA-based transformation, breeders were provided with a reliable tool to improve the crops. For the past several years, the GM crops showing resistance to viruses, herbicides and insects have led to enhanced yields and better profits to the farmers, along with a reduction in the environmental impact of agriculture within developing as well as developed nations (Barfoot and Brookes 2007; Wild and Gong 2009). The transgenic crops have found to be of benefit to especially more than 12 million small-scale farmers of the developing nations (Brookes and Barfoot 2009). The plant breeders have exploited this technology for improving the nutritional content of the crops in order to curb malnutrition and provide better health options (Chassy et al. 2008; James 2009). There has been a slow development for the nutritionally improved transgenic crops than the ones with agronomically enhanced traits. At present, transgenically modified oilseeds with increased oil content and high-lysine maize seeds are planted by the farmers, whereas transgenic crops with higher and better nutrition still await for approval or are in the development or testing phase (Chassy et al. 2008; James 2009). Also, for the transgenic crops to be distributed freely, it needs to pass a safety review before going to the market by the food safety regulator bodies (Bradford et al. 2005; Chassy 2008; Newell-McGloughlin 2008). On the contrary, there is no need of undergoing testing before being introduced in the market for the crops developed via conventional breeding methods (Cellini et al. 2004; Parrott 2005; Kalaitzandonakes et al. 2007).

11 Clinical Trials

The main risk associated with food security involves not having a healthy diet and thus failing to avoid food-related diseases. More than 1 billion of the world's population suffers from malnutrition and food insecurity. Genetically modified crops such as Golden Rice undergo rigorous safety evaluation before being introduced into the market. Safety of newly developed proteins and various other products are obtained, and in the case of observed changes, the evaluation is done with the help of animal studies and compositional analysis (Chassy 2010). Such studies result in proving the safety of the unique product in comparison to other related varieties, though such rigorous evaluation is questioned. Even if considering the possibility of risk in Golden Rice, it poses an infinitesimally little risk to consumers, while on the other hand it can be used to save a million lives every year. On the contrary, if Golden Rice would have been developed by conventional breeding techniques, which have less precision, it would not have undergone such rigorous assessment and would already be available to poor farmers. The intense regulatory process for transgenic crops is hence proving to be of an utmost drawback to the poor and hungry population.

The need for healthy and enough food supply to the world has been the primary force behind the event of innovative ideas and technologies in the food and agriculture industry (Diamond 1997). Regulatory bodies for food safety have been

developed to ensure the safety of continuous food supply, such as EFSA in Europe, FDA in the USA and likewise many other similar agencies in other nations (Chassy 2008).

12 Field Trials

Several varieties of Golden Rice have been developed since its discovery (Fig. 5). Varieties of Golden Rice are tested initially within greenhouses, and then trails are carried out in fields. In Louisiana of the USA, the first-ever field trial of Golden Rice took place in the year 2004 and 2005 (LSU 2004; Datta et al. 2007). In order to compare and research the agricultural traits like the quality of seeds, yield, and height of Golden Rice, it was grown along with non-genetically modified plants of rice. It is important to map these traits along with the nutritional enhancements as it profits the farmer which they ultimately depend on for their livelihood. Above all, the assessment of Golden Rice cultivation on the environment is also done. It is expected that provitamin A trait is not liable to cause harm to the environment as it is already prevalent within nature and is not selectively beneficial for any wild cross-able plants. The regulatory bodies perform an extensive analysis of the genetically modified crops for possible environmental risks before they can be commercialized.

Moreover, in the next 5 years, IRRI along with the Philippine Rice Institute carried out field trials on a large scale in various regions of the Philippines (IRRI 2013b). Data from such trials are obtained to request the cultivation of Golden Rice within a country. Field trials for Golden Rice started in Bangladesh, in 2015 (IRRI 2014). The first-generation Golden Rice (known as *GR1*) was developed through infusing genes from a daffodil, but later the second-generation variety (known as *GR2*) was developed by taking a gene from corn as it gave a much better expression of provitamin-A.

Six lines of *GR2* (scientifically called "events") were developed, and the IRRI chose to work on one called *GR2R*, which it developed and subsequently infused in Filipino and Bangladeshi rice varieties. After years of lab and greenhouse tests on

Fig. 5 Variety of Golden Rice (Source: Goldenrice.org)

GR2R, the Philippines and Bangladesh eventually halted the process upon an IRRI advice that Event *GR2E* would work better.

Indonesia too is joining in to introduce the Golden Rice in the markets to avoid *VAD* among its population. In 2006, International Rice Research Institute (IRRI) and its partners began working with a new version of the Golden Rice trait that produces significantly more beta-carotene than the 1999 prototype, and it is this version of Golden Rice that is still under development and evaluation.

After more than a decade of rigorous scientific testing and extensive research trials amidst opposition by anti-GMO groups, Golden Rice is closer to getting introduced in the markets of Philippines. In 2017, the IRRI and Philippine Rice Research Institute (PhilRice) submitted an application for a biosafety permit to the Department of Agriculture Bureau of Plant Industry (DA-BPI) seeking approval to allow direct use of Golden Rice (*GR2E*) as food and feed or for processing. PhilRice and IRRI worked together in the Philippines to develop Golden Rice as a potential new food-based approach to improve vitamin A status. The work involved: (a) developing varieties suitable for Asian farmers; (b) help assess the safety of Golden Rice; (c) evaluate whether consumption of Golden Rice improves vitamin A status, and (d) explore how Golden Rice could reach those most in need.

An application for commercialization or unrestricted cultivation of GR2E rice, a variety of Golden Rice in the Philippines, was submitted. The proposed use of GR2E is the production of rice for human consumption (e.g. milled rice and derived products, such as bran, germ, starch, flour and oil), as well as rice by-products for use in livestock feed. Based on documents submitted by PhilRice and IRRI, GR2E was developed using recombinant-DNA techniques to increase the amount of provitamin A (mainly beta-carotene) in the rice endosperm, which is then converted in the body to vitamin A.

According to the World Health Organization's global *VAD* database, one in every five preschool children in Bangladesh is vitamin A deficient. Among pregnant women, 23.7% suffer from *VAD*.

Upon receipts of a positive outcome from two successive years of 'confined' field trials, the breeders at the Bangladesh Rice Research Institute (BRRI) have just gone for the last cycle of multi-location field trials and had sought regulatory approval from the government for a field trial before seeking variety release approval. The BRRI dhan29, developed by BRRI in 1994, is the most productive dry season rice variety of Bangladesh that has gone beyond national boundaries to be grown in many other countries including India, China, Vietnam, Nepal, Bhutan and Myanmar. BRRI Senior Plant Breeder observed that in the last Boro season approximately 10–12 μg/g of beta-carotene was obtained in a BRRI dhan29 line genetically converted into Golden Rice, which is considered enough to address half of the rice-eating consumer's daily deficiency of vitamin A. Recently, it is speculated that Bangladeshi rice scientists have advanced the beta-carotene-rich rice to a stage very close to the release of Golden Rice. With this development, a long wait is nearly over for rice breeders who have been trying since 1999 for a varietal development and release of Golden Rice, long being touted by the scientist fraternity as a key remedy to acute *VAD* problem. BRRI scientists analysed the post-harvest

data collected from the first field test conducted on—'*GR2E* BRRI dhan29'—during the 2015–2016 Boro season and again the data generated from multi-location trials conducted in 2016–2017 Boro season concluding that the results are positive. Golden Rice was first developed by splicing three foreign genes, two from daffodil and one from a bacterium into *japonica* rice, a variety adapted to temperate climates. It is capable of producing beta-carotene. However, for a better beta-carotene expression in rice, the daffodil genes were replaced by maize genes later in 2005. None of the major diseases like a blast, sheath blight, bacterial blight and tungro was observed in the transgenic *GR2E* BRRI dhan29, and the yield was on average 10% higher than that of the BRRI dhan29 with a functional expression of beta-carotene. Although Bangladeshi rice scientists have been at the forefront of Golden Rice research since the development of this transgenic rice by Swiss and German scientists in 1999, the process gathered momentum only when then IRRI plant biotechnologist, Dr. Swapan K Datta, infused the genes responsible for beta-carotene into BRRI dhan29 in 2002–2003.

In April 2011, Seattle-based Bill and Melinda Gates Foundation sanctioned a grant of over $10 million to IRRI to fund, develop and evaluate Golden Rice varieties for Bangladesh and the Philippines. Later further funding was also made available. Officials concerned at IRRI and Gates Foundation said as the Golden Rice inventors and subsequent technology developer Syngenta allowed a royalty-free access to the patents, the new rice would be of the same price as other rice varieties once released for commercial farming in Bangladesh, and farmers would be able to share and replant the seeds as they wish. Even though it's taking time for Bangladesh to start the Golden Rice release process, two countries, Australia and New Zealand, have already cleared this biotech rice product for consumption in those countries as both food and feed, while a review is underway in the USA by the Food and Drug Administration (FDA). In 2018, Golden Rice received three successive positive food safety evaluations from leading regulatory agencies: Food Standards Australia New Zealand (22 February 2018), Health Canada (16 March 2018) and the US Food and Drug Administration (24 May 2018). Health Canada has approved the sale of a genetically modified crop that is not intended for sale in this country, and that has a GMO critic wondering what is going on. In March 2018, the federal department gave the regulatory nod to Provitamin-A Biofortified Rice Event *GR2E*, otherwise known as Golden Rice. The department believes that the changes made in this rice variety do not seem to pose a higher risk to human health than the currently available rice varieties in the Canadian market can. The International Rice Research Institute has received regulatory approval for the product in Canada, Australia and New Zealand, despite declaring it has no intention to sell Golden Rice in those markets. The institute's real goal is to achieve regulatory approval to grow and sell the rice in the Philippines, Bangladesh, India and Indonesia, but it has yet to submit applications in those markets. The regulators favour the selling of products containing traces of Golden Rice, which is genetically modified to produce beta-carotene in the markets of Australia and New Zealand. Proponents of biotechnology believe Golden Rice can help them in the publicity battle over genetically

modified crops because it is designed to help address child malnutrition in developing countries rather than controlling weeds or insects.

13 Future Prospects

After the achievement of accumulating provitamin A within the endosperm of rice, its quantity can be increased based on the choice *psy* gene and the by event selection. The basic upstream research for the development of Golden Rice has been completed. Similar researches have begun on other crops in order to fight issues related to *VAD*. A new scope has been developed in directing the research to combine other nutritional traits such as zinc, iron and vitamin E with the Golden Rice and also the deposition of essential amino acids and protein of higher quality within the endosperm of rice. One of the essential components of our diet is vitamin E which consists of tocopherols and tocotrienols. They are also helpful in stabilizing provitamin A in the matrix of the food due to their antioxidant property. In order to increase the amount of vitamin E within the rice endosperm, other transgenes need to be inserted into the Golden Rice. a-tocopherol present abundantly within the embryo of rice is removed in the milling process. Above all, there is enough information on the vitamin E pathway and its engineering along with the entire suite of vitamin E pathway genes (Ajjawi and Shintani 2004). Several proof-of-concept experiments are needed to be performed in order to determine the gene complement for the grain's endosperm and also to determine whether and how the production of vitamin E and provitamin A can be enhanced simultaneously, as both these pathways have the same precursor molecule, i.e. *GGPP*. Plants deal with severe micronutrient deficiency of iron and zinc. Moreover, there is a need to study iron metabolism thoroughly, in order to deal with the problems associated with iron and zinc accumulation. An estimated one-third of the world's population is anaemic due to the deficiency of iron (http://www.who.int/nut/ida.html).

Several transgenic methods have been employed for enhancing the content of iron, but only minor changes were observed, as the metal loading in plant's phloem transport pathway is the basis of determining the mineral content of the seed (Grusak 2002). For the involvement of iron-homeostasis within the rice, 43 genes which encode for proteins across five families are under consideration (Gross et al. 2003). It is challenging to assess multi-gene traits such as this with the help of GMO technologies and therefore needs vast proof-of-concept research to be done for determining the rate-limiting steps for the accumulation of iron within the rice endosperm. Simultaneously, several screenings of the germplasms have begun by the plant breeders for introducing variation in the seed mineral content. So far, fourfold variation has been observed in the seed for iron and zinc (Gregorio et al. 2000). Hence, this strategy can be employed for introgression of the Golden Rice trait by using the iron and zinc rice germplasm. Another work is underway for reducing the amount of anti-nutritive factors for divalent metal cations and enhancing the bioavailability promoters, thereby improving the absorption capacity of ingested zinc and iron. Based on the identification of QTLs, a clear strategy is

under investigation for determining the biochemical basis of the bioavailability of iron.

Further challenges faced involve the increased protein complement in the Golden Rice or the attainment of a balanced composition of amino acids. As of now, the quality and the content of protein in rice have not improved with the help of mutant screening or conventional breeding approaches. Although adopting GMO is straightforward in theory, previous research has shown a backlash in the trade of cereals due to decrease in yield or other nutritional components or the quality of the grain due to enhancing the levels of protein in it (Sun and Qiaoquan 2003). An alternative approach involves altering the free essential amino acid content by employing genetic engineering on their respective biosynthetic enzymes. This approach has garnered much attention over the years. However, some challenges arise like enhanced catabolic activities, abnormal phenotypes and feedback regulations (Sun and Qiaoquan 2003). Since the supply of proteins in the nutrition of humans seems to rely tremendously on the sources of a plant, and with the role of rice in this matter which is food to more than 50% of the population of the world. It is quite necessary to put in more efforts through proof-of-concept experiments via both the approaches to an effective solution to the problem would be found.

14 Conclusion

Genetically modified (GM) crops are not magic that solves all the problems among the developing nations. However, they can significantly help in decreasing poverty, provide sustainable development and provide better health and nutrition. Golden Rice has emerged as a promising second-generation genetically modified technology, which can reduce issues related to VAD at a little cost despite accounting for outlays for social marketing in the future. There has been significant progress in the Golden Rice development across the past years, and now efforts are going into making Golden Rice accessible to the farmers and the target population within the developing nations to curb the diseases against VAD.

There is a necessity of collaborating international research consortia and complementary expertise. More important is the early and immediate involvement of National Agricultural Research Institutions of the nations with VAD to not only share their complementary knowledge but additionally for the creation of the project's ownership. The two recently formed consortia include HarvestPlus (http://www.harvestplus.org), while the other one is in the Grand Challenges in the Global Health initiative (http://www.gcgh.org/subcontent.aspx?SecIDZ390). It gets the funding by Bill and Melinda Gates Foundation, involved in targeting cassava, banana, rice and sorghum via genetic modification. Consortia such as these are anticipated to form the focal points of a new type of Green revolution along with addressing the issues regarding the content of food nutrition. This issue has faced much negligence in the past years.

Also, there is a need for the public sector to resource the projects based on the developing nations. Although, the private sector potentially can play an essential role

in projects targeted for the sparse population in order to provide benefit to all the participating parties. The public-private sector and multidisciplinary partnerships along with their management need continuous funding, knowing that genetic modification is a necessity instead of an option to produce sustainable nutrition in the staple food of the developing nations.

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Biofortification of Rice with Iron and Zinc: Progress and Prospects

Usman Zulfiqar, Muhammad Maqsood, and Saddam Hussain

Abstract

Diets of more than two–third of the world population are deficient of one or more essential micronutrients. Among micronutrients, deficiency of iron and zinc is most common in staples especially in wheat and rice that leads to occurrence of serious disorders in humans especially in pregnant women and young babies. Deficiency of iron and zinc is most critical as these micronutrients are required for functional and structural integrity of biological systems in humans affecting more than half of population globally by destroying immune systems and hampering growth and development. Deficiency of essential elements can be remediated by food supplementation, dietary diversification, food fortification, and biofortification. Among these approaches, biofortification is a relatively economical and pertinent option, and it aims to overcome micronutrient deficiency by improving their concentration in edible crops that are in the reach of poor people living in remote areas. It aims to improve the nutritional status of edible parts of cereals through genetic (conventional breeding, transgenic approaches) or agronomic (application of micronutrients to plants) ways. Transgenic approaches offer the precise and rapid way to develop crops with improved nutrition, thus complementing conventional breeding and mineral fertilization toward ameliorating the scourge. Till now 0.02 billion households are getting benefits from diversified diets and consuming micronutrient-rich crops. In this chapter, we review the biofortification of rice with iron and zinc by use of agronomic practices, crop breeding, and transgenic approaches.

Keywords

Agronomic interventions · Biofortification · Iron · Micronutrient deficiency · Zinc

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1 Introduction

The erratic rise in the prices of commodities related to agriculture, recent financial crisis, and lack of dietary diversification leads to income reduction and hike the food prices (Bouis et al. 2011). This situation is negatively affecting the health of people due to low intake of foods enriched with micronutrients such as zinc (Zn) and iron (Fe), especially in rural-based people (DellaPenna 2007). Micronutrients are imperative for the well-being and health of humans and have a very vital role in the functioning of human body (Wang et al. 2011). Deficiency of these essential micronutrients results in serious health disorders such as anemia, increased death rate, impaired mental and cognitive development, reduced growth rate, and impairment in immune system (Black 2003). Malnutrition of micronutrients not only enhances the prevalence of diseases but also depreciates economic productivity and social well-being globally (Stein 2010; Akhtar et al. 2019). Presently, 3 billion people are suffering from micronutrient malnutrition evolving from iron and zinc that cause severe health consequences (Kumssa et al. 2015a, b) (Fig. 1). This deficiency causes the death of about 25,000 children daily worldwide (Anandan et al. 2011; Kirsten 2010). For instance, in England, 11–38% of children are suffering from anemia before reaching the age of 2 years. Moreover, in the United States and Canada, 10% of the total population is zinc deficient (Gregory et al. 2017). The major staple crops of most of countries of the world are wheat, rice, and maize. There are several ways to improve the concentration of micronutrients and

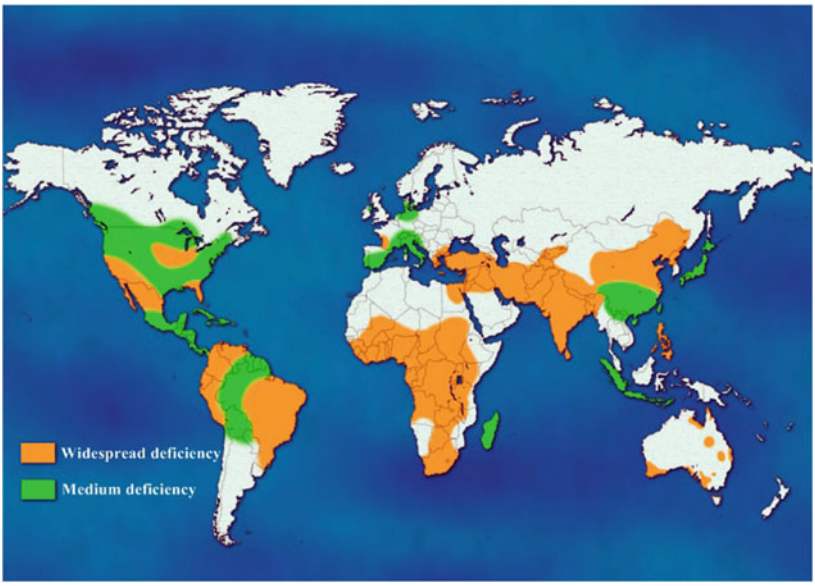


Fig. 1 Zinc concentrations in soil worldwide. Green color shows the countries moderately deficient in Zn, while orange area represents the countries having widespread deficiency of Zn (Source: Alloway 2008)

their bioavailability in edible portions of these cereals such as supplementation, dietary diversification, food fortification, and biofortification (Akhtar et al. 2019).

2 Hunger and "Hidden Hunger"

The word malnutrition is a broader term that indicates the situation that may arise as a result of overnutrition or undernutrition. Overnutrition leads to obesity and serious diet-related noncommunicable disorders such as diabetics, heart diseases, and blood pressure whereas undernutrition is defined as availability of inadequate quantity of food which could be called as hunger, or availability of adequate amount of food but with low level of nutritious and indispensable food elements that could be referred as hidden hunger. Commonly hunger is understood as a shortage of food supply to consumers, but it can also be referred as presence of sufficient food with deficiency of essential vitamins and minerals, and this hunger and hidden hunger are associated with wasting, stunting, and other serious health concerns (WHO 2016a, b). Hunger problems have been significantly reduced in developing countries as compared to the past. Since 2000, the global hunger index (GHI) has been decreased up to 29% (Anon 2016). Although situation is better from the past, still there are serious hunger threats in more than 50 countries including India, Pakistan, Indonesia, and Bangladesh owing to high population. So there is a need for serious effort to eradicate hunger and hidden hunger and to achieve zero hunger rate worldwide.

3 Options for Eliminating Micronutrient Malnutrition

There are some options to overcome the problem of malnutrition such as diversification of food, supplementation, food fortification, and biofortification (Fig. 2). All these strategies are effective and have their own pros and cons.

3.1 Dietary Diversification

The dietary requirement of the human body cannot be fulfilled by a single food. A combination of different natural foods that can supply essential nutrients needed for normal functioning of human body is known as balanced nutrition. Diversification in daily diet for long terms ensures healthy nutrition containing a feasible combination of macronutrients and micronutrients (Thompson and Amoroso 2010). Intake of fruits, vegetables, cereals, legumes, meat, and fish provides sufficient nutrition for all age and gender groups (FAO 2013).

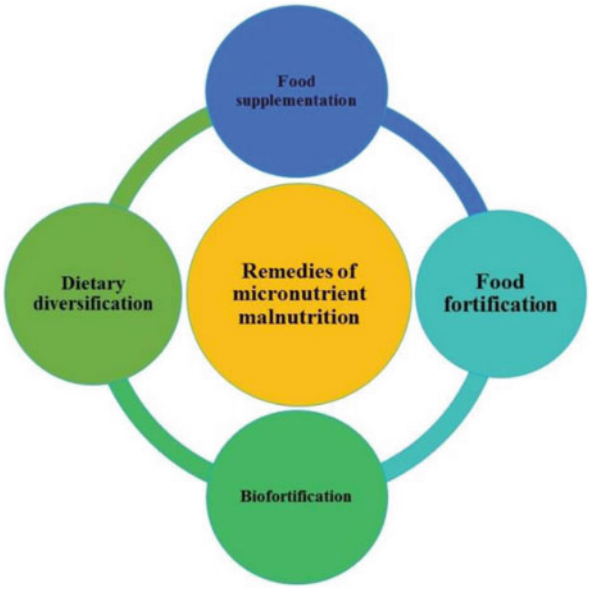


Fig. 2 Options for eliminating micronutrient malnutrition

3.2 Food Supplementation

It is the addition of essential micronutrients to the daily diet of a person to overcome the deficiencies. This remedy is effective in advanced countries worldwide where these supplements are comparatively economical in comparison with a person's income. However, use of supplements to fulfill nutritional necessities can be costly and tough to sustain because half of the population live in remote areas where people have no access to these supplements. Moreover, people living in third world countries cannot afford the expenses of supplements. The trend of use of supplements in daily diets increased considerably in the past (UNICEF 2013). More than 75% of children aged 0.5–6 years old required vitamin A twice in a year to decrease its deficiency that is related to mortality (UNICEF 2007). Supplementation of other essential micronutrients such as Fe, Zn, and amino acids is less common. Fe-folate supplements are recommended in many countries for expectant women, though it is not common in underdeveloped nations owing to low incomes. Nonetheless, WHO urges 30–60 mg per day supplementation of Fe for expecting females on day-to-day basis to avoid puerperal sepsis, maternal anemia, preterm births, and low birth weights (WHO 2016a, b).

3.3 Food Fortification

The addition of nutrients to food for improving or maintaining the food quality of an individual, group, or community is referred as food fortification. It is needed due to the inadequate intake of minerals and vitamins in conventionally processed foods.

Commercial fortification of food adds essential micronutrients in trace amounts that helps the consumers to achieve required micronutrients levels in their daily diet. The addition of iodine in table salt is a cost-effective and relatively sustainable example of fortification, and about 71% of the world population has access to iodine-rich table salt. Moreover, since 2003, the number of countries deficient in iron has reduced from 54 to 32 (Andersson et al. 2012). However, it may be more suitable and effective for consumers living in urban and developed areas, who have an easy access to commercially fortified and processed foods, but for rural people, it is hard to access the fortified foods. Various other limitations can also come in adoption of food fortification such as reluctance from people to use these processed foods, and bioavailability of essential nutrients may be decreased due to cooking. So, the active coordination among researchers, policy makers, and economists is needed for the effective use of fortified foods on long-term basis.

3.4 Biofortification

It is a process of deliberately improving the nutritional status of edible parts of plants through conventional breeding or transgenic approaches or application of essential micronutrients in soil or as foliar (Fig. 3). It mitigates deficiency of micronutrients by increasing concentrations and bioavailability of micronutrients in edible portions of staple cereals. Among the scientific community and stakeholders, biofortification is

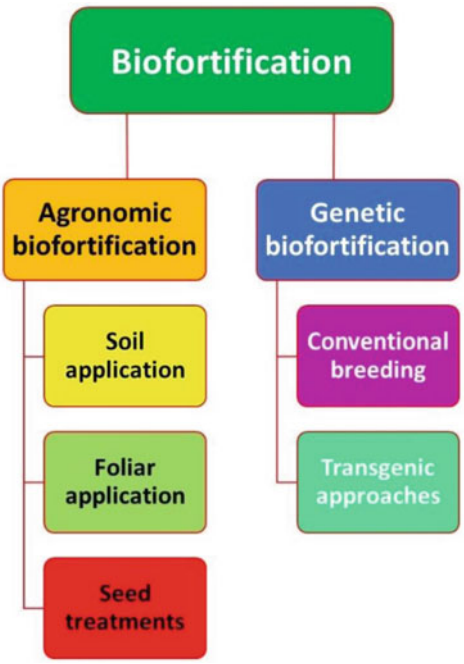


Fig. 3 Flow chart of biofortification

broadly accepted as a comparatively better approach than supplementation, food diversification, and fortification due to its effectiveness (Hotz et al. 2012). Biofortification strategy is cost-effective, and it is easy to disseminate even in rural areas of third world countries. The basic purpose of biofortification is to reduce the rate of morbidity and mortality linked with malnutrition of micronutrients and to improve the food security and quality of life of people in underdeveloped countries. Numerous biofortified crops have been developed so far comprising Fe-enriched beans, Zn-enriched rice, and Zn-fortified wheat (Tanumihardjo 2013; Talsma 2014; Akhtar et al. 2019). Improved concentrations of micronutrients in various crops such as corn, wheat, rice, beans, potato, and pearl millet have substantially improved the nutritional status of humans (Bouis et al. 2011).

4 Agronomic Interventions

Nongenetic approaches to overcome the nutritional deficiency in food plants could be more effective, and in this regard, various strategies can be adopted to enhance the concentrations of essential micronutrients in edible parts of plants (Akhtar et al. 2019). These approaches include different management practices, application of targeted element/nutrient to plants in soil, or application of theses nutrients on foliage of plants. It has been reported that agronomic biofortification not only enhances the yield, but it also improves the nutritional status of staple crops (Imran et al. 2016; de Valenca et al. 2017). Nutrient fertilization is an efficient and immediate route to improve the concentration of trace elements in crops; nonetheless, genetic biofortification may be cost-efficient in the long run. Biofortification of staple crops with vitamins is not feasible through agronomic ways, but this has enormous potential to improve mineral concentrations (Broadley et al. 2006). Agronomic biofortification aims at reducing the deficiencies of Fe and Zn in soil-plant systems and improving the nutritional quality of staple grains. Agronomic biofortification can be used as complementary measure to genetic biofortification (Cakmak 2008; Akhtar et al. 2019). Agronomic approaches are soil application, foliar application, Fe and Zn application to seeds, or combination of these (Tables 1 and 2).

4.1 Soil Application

The availability of Fe and Zn in rice depends upon redox potential of soil, as rice is commonly cultivated in anaerobic flooded conditions. Under submerged conditions, due to precipitation of insoluble zinc sulfide, Zn becomes deficient. Zinc is mostly applied before flooding or after nursery transplantation under lowland conditions in rice to avoid Zn deficiency and enhance grain yield (Dobermann and Fairhurst 2000; Naik and Das 2007). Appropriate selection of Zn source is also an important factor to improve the availability of Zn in lowland rice. $ZnSO_4$ and Zn-EDTA are better sources of Zn as compared to fritted Zn and ZnO, due to their greater solubility and

Table 1 Comparative performance of Zn application methods and sources for grain Zn concentration and grain yield of rice in different production systems

Location	Zn sources	Method of Zn application	Rate of Zn application	Production system	Rise in grain Zn concentration (%) as compared to control	Rise in grain yield (%) as compared to control	Reference
Vientiane, Laos	$ZnSO_4 \cdot 7H_2O$	Soil application	50 kg ha^{-1}	Conventional flooding	14	35.3	Nattinee et al. (2009)
D.I. Khan, Pakistan	$ZnSO_4 \cdot 7H_2O$	Soil application	10 kg ha^{-1}	Conventional flooding	60	–	Khan et al. (2003)
New Delhi, India	$ZnSO_4$-coated urea prills	Seed coating		Conventional flooding	12.8	6.8	Shivay et al. (2008)
New Delhi, India	ZnO-coated urea prills	Seed coating		Conventional flooding	4.4	19.1	Shivay et al. (2008)
Putra, Malaysia	$ZnSO_4$	Soil solution		Conventional flooding	25	–	Sharifianpour et al. (2015)
New Delhi, India	Zn-EDTA	Soil application at + two foliar sprays	2.5 kg ha^{-1} (soil application) (0.5% at foliar spray)	Conventional flooding	20.60	–	Ghasal et al. (2018)
Faisalabad, Pakistan	$ZnSO_4 \cdot 7H_2O$	Soil application	10 kg ha^{-1}	Conventional flooding	46	23.91	Farooq et al. (2018)
Faisalabad, Pakistan	$ZnSO_4 \cdot 7H_2O$	Soil application	10 kg ha^{-1}	Direct seeding	40.44	23.68	Farooq et al. (2018)
Faisalabad, Pakistan	$ZnSO_4 \cdot 7H_2O$	Soil application	15 kg ha^{-1}	System of rice intensification	5	7.2	Rehman et al. (2018)
Faisalabad, Pakistan	$ZnSO_4 \cdot 7H_2O$	Soil application	15 kg ha^{-1}	Continuous flooding	9.95	8.76	Rehman et al. (2018)

(continued)

Table 1 (continued)

Location	Zn sources	Method of Zn application	Rate of Zn application	Production system	Rise in grain Zn concentration (%) as compared to control	Rise in grain yield (%) as compared to control	Reference
Faisalabad, Pakistan	$ZnSO_4 \cdot 7H_2O$	Soil application	15 kg ha^{-1}	Alternate wetting and drying	18.6	53	Rehman et al. (2018)
Tamil Nadu, India	$ZnSO_4$	Soil application	2.5 ppm	Conventional flooding	40.54	35.2	Muthukumararaja and Sriramachandrasekharan (2012)
Phitsanulok, Thailand	$ZnSO_4 \cdot 7H_2O$	Foliar application	0.5%	Conventional flooding	33.3	3.5	Boonchuay et al. (2013)

Table 2 Comparative performance of Fe application methods and sources for grain Fe concentration and grain yield of rice in different production systems

Location	Fe sources	Method of Fe application	Rate of Fe application	Production system	Rise in grain Fe concentration (%) as compared to control	Rise in grain yield (%) as compared to control	Reference
New Delhi, India	$FeSO_4 \cdot 7H_2O$	Soil application	67 mg kg^{-1} soil	Aerobic rice	25		Meena et al. (2016)
New Delhi, India	$FeSO_4 \cdot 7H_2O$	Soil application at + two foliar sprays	50 kg ha^{-1} (soil application) (2% foliar spray)	Continuous flooding	14.82	6.5	Yadav et al. (2015)
Zhejiang, China	Fe-amino acid complex [Fe(II)-AA]	Foliar spray	0.1% (w/v)	Continuous flooding	25.80		Zhang et al. (2008)
Zhejiang, China	Fe(II)-AA + boric acid compound	Foliar spray	0.1% (w/v)	Continuous flooding	21.42		Zhang et al. (2009)
New Delhi, India	$FeSO_4$	3 foliar sprays	2.0%	Continuous flooding	16.20	4.69	Yadav et al. (2013)
Hangzhou, China	$FeSO_4$ + nicotianamine (NA)	Foliar spray	$FeSO_4$ (w/v, 0.2%) NA (w/v, 1%)	Continuous flooding	24.44	11.10	Wei et al. (2012)
Zhejiang, China	Fe-AA + NA	Foliar spray	0.1% (w/v)	Continuous flooding	50	5.72	Yuan et al. (2012)
Karnal, India	$FeSO_4 \cdot 7H_2O$	Soil application	50 ppm	Submerged conditions (pots)	26.73	1.80	Swarup (1981)
Zhejiang, China	$FeSO_4$	Seed soaking	0.5 g/L		66		Wei et al. (2013)

their efficient transport to roots (Kang and Okoro 1976). Higher Zn transport to soil boosted the possibility of interception of Zn by rapidly growing roots, which may be linked with a larger impact of Zn-EDTA banding than fritted Zn, though outcomes may differ from soil to soil or according to methods of application. Gupta et al. (1994) observed no significant differences in the production of dry matter among Zn-EDTA application methods, though the application of fritted Zn with soil mixture was found best than band placement and broadcasting. Uptake of Zn by rice was found to be effective in the following order: Zn mixing in soil > broadcasting of Zn > band placement (Giordano and Mortvedt 1973). Soil application of $ZnSO_4$ as a source of Zn has little effect on grain biofortification, but it is recommended to apply Zn to improve paddy yield. Application of $ZnSO_4$ at 2.5 ppm increased the grain yield up to 35%, and Zn fertilization improved the grain Zn concentration up to 40.5% as compared to no application (Muthukumararaja and Sriramachandrasekharan 2012). Moreover, the application of 50 kg ha^{-1} $ZnSO_4 \cdot 7H_2O$ in rice significantly improved the yield and concentration of Zn in grains (Nattinee et al. 2009). Rehman et al. (2012) carried out a 2-year experiment on rice in slightly alkaline sandy-clay soils having a sufficient amount of Zn. It was reported that the application of $ZnSO_4$ at tillering or panicle initiation improved soil and plant Zn contents significantly than application at transplanting or no application under anaerobic flooded, aerobic direct-seeded systems, and alternate wetting and drying. A rise in grain Zn concentration over basal was 2.5, 2.3, and 2.8 times in anaerobic flooded, aerobic direct-seeded systems, and alternate wetting and drying, respectively, when Zn was applied at tillering. This growth was linked with higher availability of soil Zn, improved Zn uptake in plants, and higher remobilization of Zn from leaves to grains during grain filling (Rehman et al. 2012). However, a marginal rise in grain Zn concentration was observed when Zn was applied at transplanting time of lowland rice (Srivastava et al. 1999). Even though application of Zn in soil is a promising approach for improving its concentration in tissues as well as improving the growth and productivity of rice (Khan et al. 2003), such application is not so efficient in improving the concentration of Zn in grains (Nattinee et al. 2009), and it may not be favorable economically because of higher cost. Nonetheless, it must be considered as complementary practice to improve Zn accumulation in crops. For soil and foliar application of Fe, synthetic chelates are most effective, but their adoption is restricted due to higher cost (Fageria et al. 2002). Application of Fe as $FeSO_4$ at the rate of 50 kg ha^{-1} increased rice grain yield by 9 quintal/ha as compared to control treatment (Ram Sakal 2001). Combined application of $FeSO_4 \cdot 7H_2O$ as soil application (20 kg ha^{-1}) and foliar application (2% w/v solution) in submerged rice significantly boosted grain yield and grain Fe contents (Yadav et al. 2015).

4.2 Foliar Application

Another way of agronomic biofortification is foliar application of micronutrients. Micronutrients applied to foliage are absorbed by stomata and transported through the vascular system to where they are needed (Marschner 1995). A variety of zinc

sources have been used as a foliar application in various crop plants such as Zn $(NO_3)_2$, $ZnSO_4$, and Zn-EDTA (Yoshida et al. 1970; Akhtar et al. 2019). $ZnSO_4$ application is most suitable in improving grain Zn contents and correcting its deficiency (Jiang et al. 2008; Stomph et al. 2011). Karak and Das (2006) reported that application of $ZnSO_4$ and Zn-EDTA as foliar improved grain yield and grain Zn contents, and the highest results were obtained with Zn-EDTA application. Foliar application is very effective in enhancing the Zn contents in grains (Welch 2002; Yang et al. 2007; Cakmak 2009; Fahad et al. 2015); in this regard timing of application is very important (Stomph et al. 2011; Fahad et al. 2015). Application of micronutrients including Zn and Fe through soil and foliar may be comparable in terms of yield (Yoshida et al. 1970). However, response to application methods may differ with different production systems. For example, in lowland rice, before transplanting soil application of Zn is more effective than foliar application at the rate of 0.5% w/v $ZnSO_4$ or nursery fertilization with Zn or treatment of rice seeds with $ZnSO_4$ at the rate of 2–4% w/v or dipping of rice seedlings in slurry of ZnO 2% w/v (Savithri et al. 1998). Conversely, in direct seeding of rice, foliar application of Zn found more effective in improving yield and ameliorating the deficiency of Zn (Abilay and De Datta 1978). Normally, a major improvement in grain Zn contents takes place when Zn is foliar applied at anthesis stage. Zn application to rice as foliar (0.5% w/v $ZnSO_4$) at panicle initiation stage significantly improved the whole grain Zn contents up to twofold (Phattarakul et al. 2011).

In an experiment on lowland rice under alkaline sandy clay soil, Rehman et al. (2012) reported that 0.5% w/v $ZnSO_4$ application as foliar at panicle initiation increases 1.8 times whole grain zinc contents as compared to soil application at similar stage. This rise in Zn concentration in grain was due to improvement in leaf remobilization of Zn during grain filling stage. Application of Fe in soil at the rate of 20 kg ha^{-1} proved inferior as compared to foliar application (2% unneutralized @ $FeSO_4$) in amending the Fe deficiency in rice grown on course textured soil (Das 2000). In an experiment, Zhang et al. (2009) reported that foliar application of Fe(II)-AA + boric acid compound (0.1% (w/v)) to transplanted flooded rice significantly improve the whole grain Fe contents up to 21.4% as compared to no application. Foliar application of Zn and Fe can be helpful to avoid the problem of Zn binding in the soil, but the time of application in this regard is very important, and it should be around flowering/anthesis for enhancing Zn concentration in grains.

4.3 Seed Treatments

Micronutrients can be applied to crop plants effectively through seed treatments which are efficient and economical substitute for soil and foliar application. These are applied in very small amounts, and they are directly available to germinating seed (Singh 2003). Seed treatments of micronutrients can broadly be done through coating and priming of seed (Khaliq et al. 2015; Wang et al. 2016). Seed priming is a controlled hydration method in which seeds are dipped in aerated solution of water, nutrients, or salts prior to sowing in field (Hussain et al. 2015, 2018).

Conversely, seed coating is a process in which micronutrients or other compounds are dissolved in a sticky solution to form a more or less continuous layer on the seed (Wang et al. 2016). In comparison with other ways such as soil or foliar application, seed treatments are relatively feasible and cost-effective as they are easy to operate and required in very small amount and result in improvement of germination, seedling growth, and stand establishment (Hussain et al. 2016a, b, c). Higher Zn uptake and yield was observed, when seed priming was done in Zn-EDTA solution as compared to banded or broadcast soil application, and a 0.5% w/v solution was found suitable for direct–seeded aerobic rice (Kang and Okoro 1976). Johnson et al. (2005) reported that Zn priming of rice seeds improved Zn contents of rice grain significantly, but seed priming does not directly affect yield. After carrying out a number of preliminary experiments, Johnson et al. (2005) optimized 4 mM Zn ($ZnSO_4 \cdot 7H_2O$) for rice seed priming, and primed seeds increased the Zn contents in seeds considerably, but this improvement in seed contents was not found in progeny seeds of rice. Similarly, in case of Fe, soaking of rice seeds in $FeSO_4$ solution (0.5 g/L) improved the grain Fe contents up to 66% as compared to control treatment (Wei et al. 2013). Seed coating is employed to enhance the micronutrient use efficiency by forming a concentrated formulation of micronutrients in a sticky solution in which seeds are soaked and a sticky layer is formed on seed (Singh 2007). In an experiment, Shivay et al. (2008) reported that coating of rice seeds with $ZnSO_4$-coated urea prills in conventional puddled transplanted system significantly improved the grain yield (6.8%) and whole grain Zn contents (12.8%) as compared to control. Moreover, there are no adverse effects of seed coating on the germination of rice; it can help reduce the Fe and Zn deficiency in an economical way. Seed coating of rice with $ZnSO_4$ at low concentrations was equally effective as foliar or soil application (Giordano and Mortvedt 1973). Furthermore, it was reported that coating of rice seeds with Zn-lignosulfonate, ZnO, or Zn-EDTA found more effective in panicle numbers, grain/panicle, and grain yield as compared to foliar application (Mengel and Wilson 1979). Hence, seed treatment with Fe and Zn is an advantageous and promising approach as it ensures early vigor and better stand establishment and correct micronutrient deficiency.

5 Genetic Fortification

Supplementation, fortification, and dietary diversification have major drawbacks, i.e., reduction in the concentration of micronutrients and limited stability in food items (Allen 2003; Stein 2010). These approaches require a robust system of distribution, careful implementation because of overdose side effects, and recurring expenditure (Subbulakshmi and Naik 1999; Nestel et al. 2006). There is a need to increase micronutrient content of food crops genetically (Brinch-Pederson et al. 2007). Genetic fortification has the following key advantages: long-term availability and cost-effectiveness, stability, distribution, and replication without a reduction in micronutrient concentration (Meenakshi et al. 2010; Hoddinott et al. 2012). Five key steps that can be targeted for biofortification of iron and zinc in rice are: (1) to increase

the zinc and iron bioavailability promoters in grains (2) to enhance their transport toward grains (3) to reduce the anti-nutritional factors in grains (4) to increase the uptake from soil, and (5) to improve the sequestration of zinc and iron to endosperm rather than aleurone layer and husk. Genetic engineering and crop breeding are two main approaches that can be used for genetic fortification of food crops (Bouis 2000; Johns and Eyzaguirre 2007; Tiwari et al. 2010).

5.1 Crop Breeding

Breeding for biofortification can be achieved when genetic diversity is available in primary, secondary, and tertiary gene pool of specified components in targeted crops (Mabesa et al. 2013; Fahad et al. 2015). In nutrient contents, genetic variation is often shown by plants which can be then utilized by plant breeders for the improvement of micronutrient concentrations in crops (Welch 2002; Cakmak 2008; Gelin et al. 2007). For example, fourfold variations in zinc and iron level were observed in different genotypes of rice (Gregorio et al. 2000; Grusak and Cakmak 2005). It is the most convenient method for crop improvement programs. Several national and international organizations are working to improve crop nutritional content through breeding approaches (Pfeiffer and McClafferty 2007). Sometimes there is limited genetic variation present in gene pool that can be overcome by developing breeding material through crossing of distantly related parents (Bouis 2005). Mutagenesis can also be used to incorporate new traits in commercial varieties (Lyons et al. 2005). The following scheme can be used for the achievement of biofortification goals through breeding (Fig. 4).

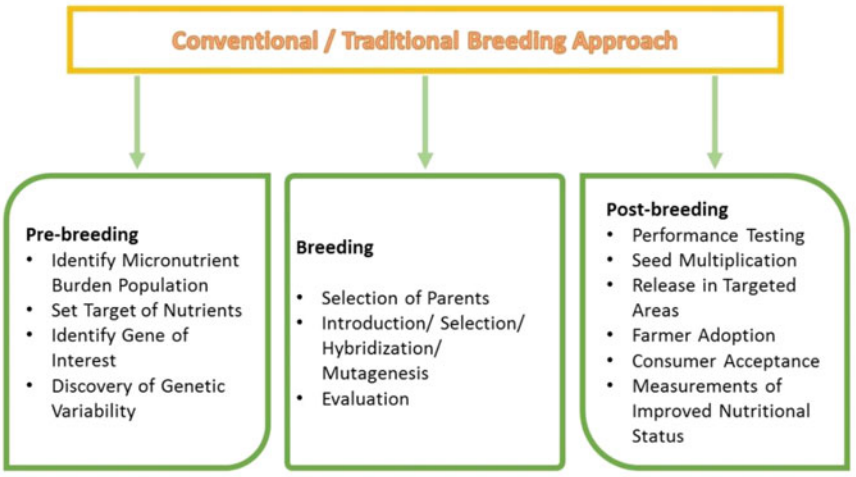


Fig. 4 Achievement of biofortification goals through breeding

Table 3 Other success stories

Type of biofortification	Status	Country/variety	Paper/ source	Content
Zinc	Released	Bangladesh BRRIdhan 62, BRRIdhan 64, BRRIdhan 72	CIAT	20–22 ppm
Zinc	Research/ traditional variety	Jalmagna	Gregorio et al. 2000	Double than common variety
Iron	Research traditional variety	Jalmagna, Philippines, India	IRRI Gregorio et al. 2000	21 ppm

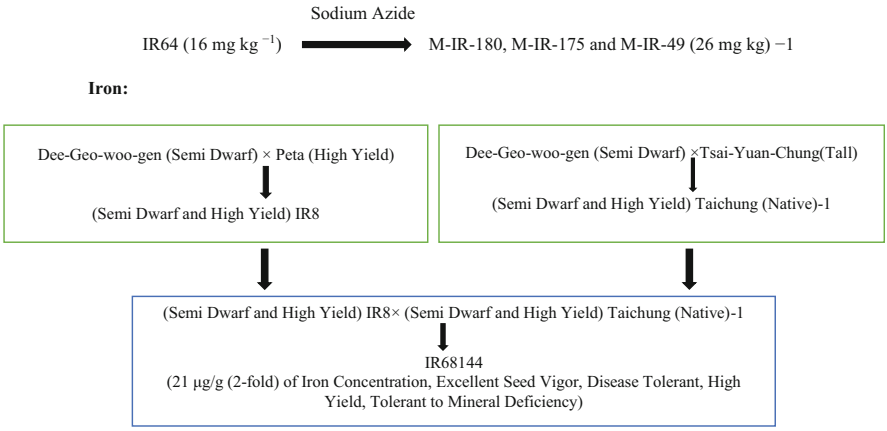
Rice through Breeding Approach

Zinc

Mutation breeding is important strategy for the improvement of concentration of zinc in rice. Mutants with high zinc content have been identified using chemical and physical mutagens (Jeng et al. 2012) (Table 3).

$$\text{IR64}\ \left(16\ \text{mg kg}^{-1}\right) \xrightarrow{\text{Sodium Azide}} \text{M-IR-180, M-IR-175 and M-IR-49}\ \left(26\ \text{mg kg}^{-1}\right)$$

Iron



5.2 Genetic Engineering

Genetic engineering and genetic transformation is best suitable when there is unavailability of genetic diversity (Zhu et al. 2007; Brinch-Pederson et al. 2007). Transgenic approach is independent of taxonomic and evolutionary status of plants; even synthetic genes can be constructed and incorporated in targeted crops. Key for developing transgenic crops is the characterization and identification function of genes and then utilization of these genes to engineer metabolism of plant (Shewmaker et al. 1999; Yang et al. 2002; Newell-McGloughlin 2008). It is feasible when targeted micronutrients do not exist in crops (Perez-Massot et al. 2013). It is fast and can be used for simultaneous gene incorporation. During the developmental stage and research, it requires substantial efforts as well as time and investment, but it is sustainable and cost-effective in the long run (White and Broadley 2005; Hirschi 2009; Hefferon 2016). Certain key steps can be followed to make a transgenic plant (Fig. 5).

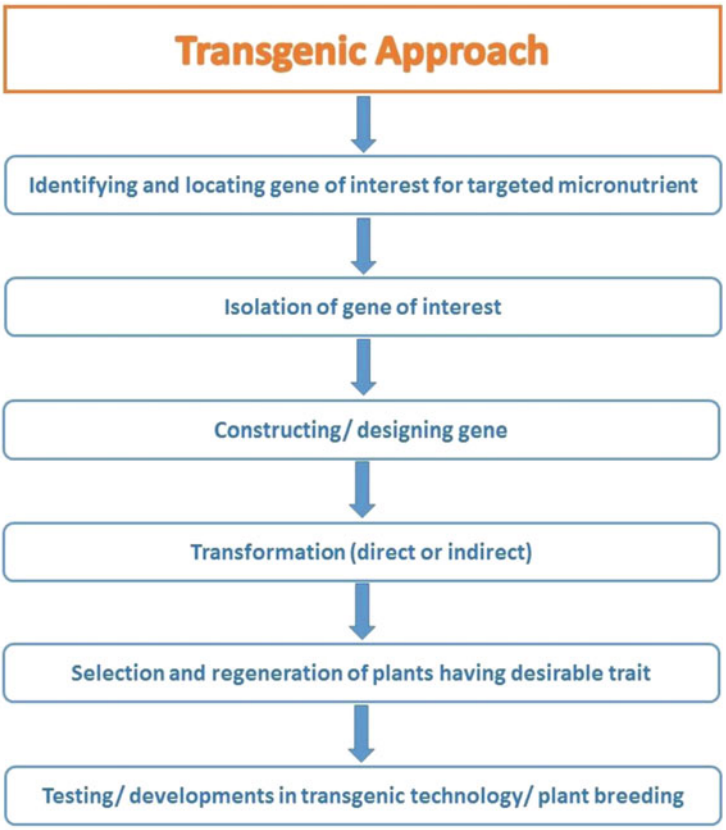


Fig. 5 Key steps to make a transgenic plant

Success Stories of Iron and Zinc Biofortification in Rice Through Transgenic Approach

Different strategies can be used for iron and zinc biofortification in rice using transgenic approach.

1. Expression of (SoyFerh1) soybean ferritin gene using (OsGLUB1) rice glutelin promoter which is endosperm specific.
   Example: In japonica cv. Kitaake, indica cv. IR68144, japonica cv. Taipei 309, and indica cv. PusaSugandhi II, iron content was increased 2-fold, 3.7-fold, 2-fold, and 2.1-fold, respectively (Lucca et al. 2001; Vasconcelos et al. 2003; Paul et al. 2012).
   Plants with ferritin gene showed 1.54-fold more zinc content (Paul et al. 2012).
2. Zinc and iron transporter gene MxIRT1 from apple tree to generate transgenic rice.
   Example: threefold increased zinc content was observed in rice plants having MxIRT1 gene. This gene could be an effective mean to biofortify rice for zinc content (Tan et al. 2015).
3. Overexpression of mugineic acid synthesis gene, nicotianamine synthase gene (NAS), and dioxygenase gene (IDS_3).
   Example: In polished rice, japonica cultivars Nipponbare, Tsukinohikari, and Dongjin showed more than threefold iron content. Japonica rice Tsukinohikari showed 1.4-fold increased iron content in polished rice (Masuda et al. 2009; Lee et al. 2009; Johnson et al. 2011).
   In rice endosperm, transgenic plant showed twofold more zinc content (Banakar et al. 2017).
4. Insertion of $OsYSL_2$ gene under control of ($OsSUT_1$) sucrose transporter promoter.
   Example: In japonica cultivar Tsukinohikari, there was 4.4-fold increase in iron concentration in polished grains (Ishimaru et al. 2010).
5. Combination of first three strategies, generating "FER-NAS-YSL2" rice.
   Example: In polished rice of japonica cv. Tsukinohikari and japonica cv. Paw Yin San, iron content was increased 4- to 6-fold and 3.4-fold, respectively (Masuda et al. 2012; Aung et al. 2013).
6. Increasing Iron content as well as increasing tolerance to iron deficiencies. There was concurrent insertion with SoyFERH2 gene and OsGLB and OsGLUB1 promoters and also mugineic acid synthase (IDS3) gene of barley and HvNAS1 nicotianamine aminotransferase genes.
   Example: In polished rice, there was 2.5 to 4-fold accumulation of iron as well as tolerance to its deficiency (Masuda et al. 2013) (Table 4).

Table 4 Other success stories

Type of biofortification	Status	Content	Paper/source	Genes
Iron	Research	14 ppm	IRRI Lee and An (2009); Takahashi et al. (2001); Zheng et al. (2010); Goto et al. (1999)	Iron transporter $OsIRT_1$ Nicotianamine synthase 1 ($OsNAS_1$) and 2 ($OsNAS_2$)
Zinc	Research		Masuda et al. (2008); Lee and An (2009)	$OsIRT_1$

6 Conclusions

Recent development in research concludes that increase in micronutrient concentration in edible parts of staple crops can be retained during cooking and processing, and after intake by humans, the nutrient present in food remains bioavailable. Biofortification is the most appropriate, proven, and feasible option to combat malnutrition, particularly for those poor people in developing countries who live in remote areas. A number of biofortified crops have been developed and introduced to overcome micronutrients malnutrition in humans. Fertilization of micronutrients, conventional breeding, and genetic engineering are tools of biofortification. To date, wheat, rice, maize, potato, beans, and pearl millet have been biofortified. To enhance the concentration of micronutrients in edible crops, research focus should be on the integration of agronomic practices and genetic strategies to improve transport of mineral to phloem-fed tissues and identification of mechanisms influencing homeostasis of minerals in plant cells. In crux, using of different strategies in a combination, improving crop efficiency to uptake nutrients and increase the vitamin/nutrients production by the use of conventional breeding and transgenic approaches along with fertilization of targeted nutrient through soil application, foliar application, or seed treatments should be considered.

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Biofortification of Iron, Zinc and Selenium in Rice for Better Quality

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Abstract

Rice is one of the most widely consumed affordable foods in the world that fulfils energy demands of the world's poor. However, common rice lacks optimum dietary concentrations of essential micronutrients which are removed during polishing. The aleurone layer in rice grains causes its deterioration during storage, so polishing is done which is coupled with the loss of nutritionally important micronutrients. Biofortification is the enhancement of essential nutrients in staple foods to combat malnutrition. Several biofortification techniques have been proved successful in improving nutritive value of rice including fertigation and foliar spraying of micronutrients and conventional rice breeding and through genetic modification. This chapter is dedicated to biofortification of rice with iron, zinc and selenium that play vital roles in important biological functions.

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1 Introduction

Rice (*Oryza sativa* L.) has crucial role in human nutrition by providing half of the dietary energy to the poor in developing countries and ~21% of global human per capita energy. It is an easily accessible and affordable food and also a primary source of income and employment for world's poor (Muthayya et al. 2014). However, it is not a perfect diet due to its insufficiency in essential nutrients which are lost during polishing. By composition, major portion of rice dry matter consists of starch (~90%) and some proteins (Champagne et al. 2004). Further, rice grain contains aleurone layer which is rich in oil and causes grain's decay during storage, making rice unsuitable for human consumption. To remove aleurone layer, rice grains are polished; however, valuable micronutrients, concentrated in husk, aleurone and embryo, are lost. This causes deficiency of micronutrients in polished-rice grains (Kennedy and Burlingame 2003).

Conventional rice cultivars have 2 μg/g Fe in polished grains which is far less than recommended daily intake of Fe for humans, i.e. 8–18 mg and are commended daily intake of 30 mg for pregnant women. Similarly, Zn density in rice varieties averages at 16 μg/g, while its recommended daily intake for humans ranges between 23 and 45 mg. Further, daily recommended intake of Se is 40–75 mg/day, while differential Se concentration in different rice varieties has been reported in the literature (Williams et al. 2009). This demands urgent steps to enhance rice's nutritive quality to cover daily intake requirements of people for Fe, Zn and Se and to avoid malnutrition.

Approximately over 2 billion population of the world is micronutrient deficient (Ramakrishnan 2002). Inability to enrich diet with essential micronutrients and vitamins has intensified the problem of undernutrition in poor populations across the globe. Human metabolic activities require several micronutrients, deficiencies of which is known as "hidden hunger". For example, deficiency of Fe, vitamin A and iodine (I) are well-documented among poor populations across the world, followed by other micronutrients. Supplementation and fortification of foods are two possible solutions to cover up micronutrient deficiency. Supplementation involves use of pills or minerals to meet instant needs of people, while fortification is the addition of micronutrients directly to the foods. However, overdosing and less bioavailability of micronutrients may be some constraints in humans. Biofortification is the process of enhancing the concentration of vitamins and minerals in cereal crops through agronomic practices, plant breeding programs and transgenic techniques. Thus, biofortified rice, wheat and maize, when consumed regularly, may make computable developments in human health and nutrition. It's a long-term strategy to improve mineral density in staple foods through fertigation and foliar application of

micronutrients, cross-breeding between compatible cultivars and genetic transformation. Besides this, increasing the bioavailability of microelements in soil and crops is also desirable in biofortification programmes. However, bioavailability of minerals can only be influenced by plant breeding and genetic engineering techniques. Although plant breeding and genetic engineering differ in scope, the two procedures are similar in aim.

Since crop's enrichment with micronutrients is easily achievable via agronomic interventions, management of soils for better plant nutrition is crucial to improving human diet. This demands use of appropriate mineral fertilizers in optimum doses, applied through suitable methods. Most importantly, these mineral fertilizers, in addition to major nutrients like NPK, should be applied at suitable growth stage of crop. This will be helpful not only to improve the density of microelements in common rice cultivars but yield also in a sustainable way.

It is hoped that biofortification of rice with essential micronutrients will contribute to improvement in the nutritional quality of rice and to eradicate widespread malnutrition. This chapter extends to discuss various strategies used for the biofortification of micronutrients (iron, zinc and selenium) in rice for better quality.

2 Biofortification Strategies to Increase Fe, Zn and Se Contents in Rice

Biofortification of rice with Fe, Zn and Se has been achieved through various approaches. Three major strategies, i.e. fertigation and foliar spray, conventional plant breeding and variety selection and genetic approaches, are discussed here in detail.

2.1 Fertigation and Foliar Spraying

Fertilizers are routinely added to the soils to increase crop yields. This practice, within certain limits, can be applied for enhancing the density of micronutrients in cereal grains (Rengel et al. 1999). Several agronomic practices including fertigation, foliar spraying, seed treatment and seedling dipping may be used in biofortification of rice. The fertigation refers to fertilizer application via irrigation water, broadcast and placements. Iron fortification in rice can be easily achieved by fertigation and foliar spraying of Fe in various forms, for example, ferrous ammonium sulphate ($(HN_4)_2Fe(SO_4)_2{\cdot}6H_2O$), ferrous sulphate ($FeSO_4{\cdot}7H_2O$) and Fe chelates such as Fe-DTPA, Fe-EDTA, Fe-EDDHA, etc. However, the bioavailability of Fe in soil is dependent on its ionic form, plant uptake strategy and soil conditions. Iron exists in soil as ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions. Under alkaline conditions, available Fe is converted to ferric oxides which are insoluble. Further, Fe uptake in plants is based on two common strategies, namely, chelation-based strategy and reduction-based strategy (Ishimaru et al. 2006; Wirth et al. 2009). Graminaceous plants follow chelation-based strategy while non-graminaceous plants follow reduction-based

chelation. Rice may adopt both strategies for Fe uptake. Moreover, soil pH, aeration, moisture and composition may also influence Fe availability and uptake by plants (De Valença et al. 2017). Especially, soil pH and aeration determine the dominance of reduced or oxidized forms of Fe in the soil. Usually, ferric ions have less solubility in the soil solution, which makes it unavailable for plant uptake. However, soil-based Fe application is not that much effective in rice due to prevalence of reduced soil conditions that rapidly convert Fe into less available form. Contrarily, foliar spraying is more suitable as Fe enters via leaf epidermis and is diverted to grain through phloem. Earlier research revealed that 0.1% $FeSO_4{\cdot}7H_2O$ foliar spray increased iron concentration in polished rice grains by 18.9% (Jin et al. 2008). In another study, average Fe content was enhanced by 32.5% in brown rice cultivars when treated with 0.1% $FeSO_4$ conjoined with amino acids and 1% nicotianamine (Yuan et al. 2013). Similarly, in a field experiment, Fe was sprayed on rice during anthesis stage in four different forms (0.2% w/v), i.e. $FeSO_4{\cdot}7H_2O$, EDTA-FeNa, HEDTA-Fe and DTPA-Fe, and the DTPA-Fe was found the best option for biofortification of rice with Fe (He et al. 2013).

Zinc is deficient in 50% of the agricultural soils that affects one-third of the population globally (Alloway 2008a). Zinc fertilizers may be applied to cover Zn deficiency in soils and crops. Zinc fertilizers are divided into four main types, i.e. (1) natural organic complexes (e.g. Znpolyflavonoid, Znlignosulfonate); (2) inorganic complexes, e.g. ammoniated Zn sulphate solution; (3) inorganic compounds such as Zn sulphate heptahydrate, Zn oxide(ZnO); and (4) synthetic chelates (Mortvedt and Gilkes 1993). Application of Zn fertilizers can be done alone or in combination with macronutrients (NPK).The biofortification efficiency of Zn fertilizers depends on their solubility and application method (Rehman 2014; Velu et al. 2014). Research has revealed that zinc sulphate and zinc chelate (Zn–EDTA) have greater solubility and efficient plant uptake as compare to zinc oxide and fitted-Zn. Moreover, chelated-Zn has higher bioavailability than $ZnSO_4$ in some soils, but it is 4–5 times more expensive than later (Alloway 2008b; Boawn 1973; Karak et al. 2005). Chelation of Zn ion (Zn^{2+}) with an organic chelating agent such as $EDTA^{4-}$ revert its charges as $ZnEDTA^{2-}$ which results in the reduction of electrostatic attraction on adsorption sites in soil. By this strategy, Zn is less adsorbed on soil colloids, leaving more available Zn for plant uptake (Zhao et al. 2012).

Although soil application of Zn is a promising method of Zn fortification in plants, it is not considered economical due to higher prices of chelated-Zn. Foliar application is more suitable as it reduces loss of nutrients with irrigation water. As foliar spray, Zn may be applied as zinc sulphate ($ZnSO_4$), zinc nitrate ($Zn(NO_3)_2$) and chelated-Zn (Zn-EDTA) (McBeath and McLaughlin 2014; Sharifianpour et al. 2015). Among these, Zn-EDTA is considered most effective as it adds to grain Zn and reduces chances of Zn adsorption on soil particles (Benedicto et al. 2011). Further, it is suggested that Zn should be applied at flowering stage for achieving maximum Zn concentration in the rice grains (Pandey et al. 2013).

The Se concentration in rice is generally low which hardly fulfils dietary requirements of humans (Williams et al. 2009). Food supplementation or biofortification is thus essential to avoid Se deficiency. However, direct use of Se,

as supplement, may result in excessive Se intake, leaving biofortification as a single viable option (Hartikainen 2005). Different Se fertilizers such as selenomethionine, sodium selenate, sodium selenite, etc. may be used to fortify rice and other crops with Se. However, the efficiency of Se fertilizer is dependent on soil conditions, concentration of Se in soil and application method. Under flooded conditions, Se fertilizers don't perform well as compared with upland conditions. Moreover, Se uptake by plants is directly correlated with the amount and form of Se in the soil. It has been suggested that Se present in the soil as selenite is transported by sulphate through the root cells, while selenite uptake is activated by the phosphorus transporter (Winkel et al. 2015). As foliar spray, both selenite and selenate are commonly practiced. In the cultivated aerobic soil, selenate from most dominantly prevails and has the potential to be taken up by the plant. While in anaerobic, paddy and acidic conditions, the selenite form commonly exists (White 2015). Foliar spray of Se has shown better results for various crops, and more recovery of Se was observed in cereals (Renkema et al. 2012).

2.2 Conventional Plant Breeding and Variety Selection

Contribution of plant breeders to making rice a high yielding staple crop and easily accessible to poor is remarkable and extended over thousands of years. However, limited attention was paid in the past to improve nutritive value of rice as main emphasis was laid on improving yield only. Plant breeding is a successful technique to integrate several limiting microelements in the rice grains as genotypic changes remain stable in the succeeding generations under changing environmental conditions. It is not only helpful to enhance the micronutrient density in rice grains; healthy seedlings are also produced in the coming generations. Ideally, once rice is fortified through plant breeding program, farmers can grow rice indefinitely without any additional costs. That's why, biofortification through plant breeding is an encouraging and cheap strategy (Bouis and Saltzman 2017; Poletti et al. 2004; Sharma et al. 2017).

Researchers from several countries of the world like the Philippines, Thailand, China and India have conducted rice breeding programs for the biofortification of rice with Fe (Khush 1987). The emphasis is on selecting rice genotypes that are resistant to excess soil Fe. Researchers have observed that great genetic variability prevails among rice cultivars for iron toxicity (Audebert and Fofana 2009). In the Philippines, researchers have found that "CK4" was a more Fe-tolerant cultivar under flooded conditions due to its better photosynthesis and less Fe density in leaves (Audebert and Sahrawat 2000). Comparatively, rice grain has less iron contents than legumes (Frossard et al. 2000). Therefore, sufficient potential for biofortification, although dependent on genetic variability, exists among rice cultivars. Historically, traditional rice cultivars have more iron density as compared to modern-day cultivars (Gregorio et al. 2008, 2000). The reason is that earlier breeders had paid more attention to increasing yield than Fe accumulation in rice grains (Zuo and Zhang 2011).

Differential uptake and translocation of Zn corresponds to genotypic variability among different rice varieties for Fe use efficiency. Earlier research has revealed that phytosiderophores, as root exudates, are released to combat Zn deficiency (Singh et al. 2005). Release of phytosiderophores stimulates mobilization of Zn and subsequent storing in grains. Moreover, rice cultivars differentially respond to Zn deficiency induced by elevated pH caused by bicarbonate contents in the soils (Forno et al. 1975). In an earlier study, though 5–10 mM bicarbonate hindered root growth in Zn-inefficient rice, the same had positive effect on growth of Zn-efficient plants, suggesting greater tolerance of Zn-efficient plants to increased bicarbonate contents in the soil (Yang et al. 1994). Similarly, Zn-efficient rice cultivars had higher ability of Zn translocation to the grains than Zn-sensitive cultivars (Somayanda et al. 2013). Previously, research has been focused on identifying major quantitative trait loci (QTLs) associated with Zn in cereal grains which may be helpful to better understand Zn uptake, its transportation and remobilization (Palmgren et al. 2008). Some QTLs associated with phytate—an anti-nutritive substance, Zn, Fe and Se accumulation in rice grains, have been identified (Norton et al. 2014; Stangoulis et al. 2007). Results showed that QTLs for phytate and micronutrients were located on different chromosomes, making possibility of altering the concentration of phytate in the grains without compromising the density of micronutrients.

2.3 Genetic Approaches

Genetic engineering is a more successful and rapid technique than conventional plant breeding for fortifying plants with essential micronutrients. Genetic engineers can transform plants with desirable genes isolated from any source, sexually compatible or incompatible or even artificial ones. Genetic engineering does not involve sexual propagation, whereas plant breeders have to select sexually compatible plants to inherit desirable traits in the plant's germplasm by growing plants in several generations. Further, genetic engineering is more target-oriented, and genes encoding synthesis of specific proteins involved in micronutrient accumulation are overexpressed. However, both strategies require initial investment on research; thereafter nutritionally enriched plants may be grown indefinitely in a sustainable way. Moreover, genetically modified crops have shown more tolerance to stress conditions and produce greater yield (Frossard et al. 2000).

Rice engineering, in biofortification perspective, has mainly been focused on transformation of genes related to increasing density of micronutrients in the endosperm, exploiting pathways that play roles in the bioavailability of micronutrients and inhibiting synthesis of anti-nutritional substances such as phytate. One of the major breakthroughs in rice engineering is the development of "Golden rice", in which vitamin A fortification was done by targeting β-carotene biosynthetic pathway (Beyer et al. 2002). Three genes involved in β-carotene synthesis were isolated from daffodil (*Narcissus pseudonarcissus*) and a bacterium (*Erwinia uredovora*) and transformed into rice's embryo via *Agrobacterium*.

In case of Fe, main focus of rice engineering has been on the overexpression of single or multiple genes involved in the synthesis of proteins that play roles in Fe uptake, its translocation and storage in rice's endosperm. For example, genes expressing nicotianamine synthase (NAS) and ferritin are involved in enhanced Fe uptake and Fe storage, respectively. Their overexpression has enhanced Fe density in rice's endosperm (Wirth et al. 2009). Similarly, Fe deficiency stimulates induction of various members of NAS family of genes in rice such as *OsNAS1*, *OsNAS2* and *OsNAS3* which play role in metal transport. In a previous study, their constitutive expression produced 14.5 μg/g Fe (4-fold) in rice (Johnson et al. 2011). In another study, in addition to ferritin and NAS genes, overexpression of *OsYSL2*, a nicotianamine transporter, exhibited 6-fold increase in the Fe density of grains under greenhouse and 4.4-fold increase under field conditions, respectively (Masuda et al. 2012). In a recent study, *AtIRT1*, an iron regulator transporter from *Arabidopsis thaliana*, either expressed alone or in combination with *AtNAS1* and ferritin, significantly improved Fe concentration in transgenic rice (Boonyaves et al. 2016). Further, root exudates of rice contain very small quantity of phytosiderophores that help chelate Fe in the soil. Genetically engineered rice has demonstrated enhanced phytosiderophores synthesis to cope with the Fe deficiency (Ishimaru et al. 2006).

Fortification of rice with Zn follows similar transgenics as discussed for iron. In a previous research, gene encoding phytosiderophores synthesis, *IDS3*, has improved Zn uptake and its concentration in rice grains (Masuda et al. 2008). However, Zn uptake in rice is under the influence of several *ZIP* transporters and a yellow stripe 1 gene, *YS1* (Ishimaru et al. 2011). For example, *OsIRT1*, an iron transporter gene enhanced iron and zinc levels in rice (Lee and An 2009). Similarly, overexpressing ferritin gene, *Osfer2*, under the influence of endosperm-specific promoter, *OsGluA2*, exhibited 1.37-fold increase in the rice Zn density (Paul et al. 2012). Further, a transgenic rice that overexpressed soybean ferritin gene accumulated more Zn in the brown and polished rice grains (Vasconcelos et al. 2003). Likewise, enhanced expression of *OsNAS2*, a nicotianamine synthase gene, increased Zn concentration in the rice grains by 2.7 fold (Lee et al. 2011).

Selenium fortification through genetic modification is a less studied subject in the rice engineering. Selenium is not essential for metabolism in higher plants, however mandatory for human metabolic activities. Its deficiency in agricultural soils is widespread, so in the plants and humans, needing Se biofortification in major cereal crops like rice. Detection of quantitative trait loci (QTL) for Se was a major milestone in the rice biofortification with Se (Norton et al. 2010). Similarly, a selenocystein emethyltransferase gene that increased Se density in *Arabidopsis thaliana* may be tested in rice also (Pilon-Smits and LeDuc 2009). Further, research may be focused on studying Se dynamics in rice under the influence of various promoter substances such as ascorbate, β-carotene, etc.

3 Conclusion

Rice is an inevitable human diet, but the cruelty of widespread malnutrition caused by the micronutrient deficiency requires improvement in its nutritional quality. It appeals for adopting appropriate biofortification strategy such as addition of micronutrients to the soil or foliar spraying, development of rice cultivars by target-oriented breeding programs and increasing the density and bioavailability of micronutrients via genetic engineering. This may pave the way to make rice a perfect diet for everyone.

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Micronutrient Biofortification in Rice for Better Quality

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Abstract

Micronutrient enhancement via biofortification is the only tool to reduce malnutrition of rice. The nutritional value of rice, especially iron, zinc, and selenium are usually low in many consuming communities. The aromatic rice cultivars have consistently higher concentration of iron and zinc in grain than the non-aromatic types. Zinc has multiple roles in the human body including the efficient functioning of cellular metabolic activities and stimulation of the immune system. Selenium is an essential element for human health but its intake is low. Accordingly, biofortified rice with this trace element can be prophylactic to consumers. Micronutrient deficiencies, especially those raised from selenium, zinc, and iron, pose serious human health problems for more than 2 billion people worldwide.

Keywords

Rice (*Oryza sativa* L.) · Micronutrients · Zinc · Selenium · Iron · Biofortificaion

1 Introduction

Rice is the most important food crop and is a staple food of the world's population. Rice productivity, quality, and profitability have become an integral part of nutritional food system (Krishnaswami 1998; Mandal and Mandal 1986; Marschner 1995, 2012; Mäser et al. 2001; Mabesa et al. 2013). Macro- and micronutrients are considered very important factors that limit plant biomass and productivity in many ecosystems (Turk and Tawaha 2001; Tawaha and Turk 2002a, b; Turk and Tawaha 2002a, b; Tawaha et al. 2003; Turk et al. 2003a, b, c; Tawaha and Turk 2004; Turk and Tawaha 2004; Abebe et al. 2005; Abera et al. 2005; Al-Tawaha et al. 2005; Al-Kiyyam et al. 2008; Al-Ajlouni et al. 2009; Al-Tawaha et al. 2010; Al-Juthery et al. 2018). The micronutrient malnutrition in rice is a common phenomenon due to deficiency of selenium (Se), iron (Fe), zinc (Zn), iodine (I), and vitamin A that may cause lower resistance to diseases in children and reduce the probability of child survival at birth (Shivay et al. 2008; Shehu and Jamala 2010; Takahashi et al. 2012; Sharma et al. 2013; Shahzad et al. 2014; Thilakarathne et al. 2014). Micronutrient concentration in grain can effectively be enhanced by application of appropriate mineral forms. The sources of micronutrients are inorganic, synthetic chelates or natural organic complexes (Cunningham et al. 1994; Chen et al. 2000; Carl et al. 2007; Högy and Fangmeier 2008; Högy et al. 2009; Boonchuay et al. 2013; Das and Green 2013; Imran and Asad 2015). It has been reported that the magnitude of yield response as well as Zn, Se, and Fe uptake by rice was enhanced with application of compost. Organic amendments, especially FYM, increase the concentrations of many nutrients and can be seen to enhance the nutritional value and nutrient balance of plant foods (McNair et al. 1981; Katyal and Randhawa 1983; Römheld 1991; Juliano 1993; Kochian 1993; Koch et al. 1996; Ruel and Bouis 1998; Kennedy et al. 2002; McDonald et al. 2002; Kant et al. 2012; McGrath and Lobell 2013;

Sadeghzadeh 2013; Khan et al. 2015). Organic acids such as citric, malic, oxalic, and phenolic that form Fe complexes are released when organic matter decomposes. In human nutrition terms, necessary for human health, bioavailability is commonly defined as the amount of a nutrient in a meal that is absorbable and utilizable by the person eating the meal (Grotz et al. 1998; Wolfgang and Bonnie 2007; Wissuwa et al. 2008; Graham et al. 2012; Hoekenga 2014; Imran et al. 2015b). The total amount of a micronutrient in a plant food does not represent the actual micronutrient content of the food that is utilizable by the consumer. This quantity must be determined independently using methodologies especially developed for such purposes. Micronutrient malnutrition is a leading health-care issue in the world today and much more detectable in developing countries (Graham and Rengel 1993; Foster and Samman 2010; Gao et al. 2011; Myers et al. 2014; Raliya et al. 2015). Selection of nutritionally rich (aromatic) varieties of rice can form the basis for a food-based solution to the nutrition needs of the population. Micronutrient malnutrition is of great public health importance in several parts of the world, especially the developing and underdeveloped countries (Fan et al. 2001; Fageria et al. 2011, 2012; Impa et al. 2013a; Fernando et al. 2014; Imran et al. 2015c). It has been estimated that about 2 billion people, about one third of the world's population, are deficient in one or more mineral elements. Although required in traces, these mineral elements are involved in many vital metabolic functions. Micronutrient deficiencies in humans can be remedied through food diversification, mineral supplementation, food fortification, and biofortification (Johnson-Beebout et al. 2009; Johnson 2013; Shivay et al. 2015; Imran et al. 2017; Imran 2018; Sundaria et al. 2018). Biofortification is the strategy of increasing the content of bioavailable nutrients in the edible parts of staple food crops for better human nutrition (IRRI 2012). Staple crops such as maize, rice, and wheat provide most of the calories for low-income families around the globe. Micronutrient deficiencies, especially those arising from Se, Zn, and Fe, pose serious human health problems for more than 2 billion people worldwide (Johnson-Beebout et al. 2009; Johnson et al. 2011; Johnson 2013; Shivay et al. 2015; Imran et al. 2017; Imran 2018). Biofortification is a proven strategy to combat micronutrient deficiency in large populations, particularly for those living in developing countries. However, to make it more effective, efficient, and acceptable for people, better planning, implementation, monitoring, and evaluation of biofortification programs are needed to produce cost-effective and socially acceptable biofortified food crops (Yang et al. 1998; Yoneyama et al. 2015). Food safety, quality assurance, and legal framework also need to be considered while developing any biofortification strategy.

2 Malnutrition Is a Global Perspective

Zn has multiple roles in the human body including the efficient functioning of cellular metabolic activities and stimulation of the immune system (Van-Oosten and Besford 1996; Trumbo et al. 2001; Wang et al. 2011b; Thilakarathne et al. 2014; Verma et al. 2016;). Zn is also present in nearly 300 enzymes in the human body and

is important for bone mineralization; the growth of body tissues and the fetus; sperm production and fertility; smell, vision, taste, and appetite; healthy growth of skin, hair, and nails; as well as blood clotting and wound healing, functioning of the immune system and thyroid, cell division, and protein and DNA synthesis (Sandström and Lönnerdal 1989; Römheld 1991; Ruel and Bouis 1998; Salunke et al. 2011; Sadeghzadeh 2013). Daily intake of Zn is important as the mammalian body has limited Zn stores, and the daily requirement is influenced by gender and physiological stage.

Zn deficiency is recognized as one of the major nutrient disorders in humans, and its effects are more profound in children (McNair et al. 1981; McGrath and Lobell 2013; Meng et al. 2014; Myers et al. 2014, 2015; Raliya et al. 2015). Zn deficiency is responsible for the development of a large number of illnesses, diseases, and some adverse conditions including stunting of growth, compromised immune system function, cancer, susceptibility to infectious diseases, Fe deficiency anemia, poor birth outcomes in pregnant women, hair and memory loss, skin problems, weakening of body muscles, infertility in men, and pneumonia in children (Mandal and Mandal 1986; Marschner 1995; Krishnaswami 1998; Mabesa et al. 2013). Impaired Zn homeostasis is associated with several diseases, including diabetes mellitus and zincuria which is one of the symptoms of diabetes. Zn supplementation amends glycemia in both type 1 and type 2 diabetes. Zn can be supplemented through dietary sources such as seafood, meat, green leafy vegetables, and grains (Katyal and Randhawa 1983; Koch et al. 1996; Kennedy et al. 2002; Khan et al. 2015). However, maintaining a sufficient Zn concentration in rice grain is important for more than half of the world population for whom rice is the staple diet.

3 Human Health and Biofortification of Micronutrients (Se, Fe, and Zn)

Se is an essential element for human health but its intake is low (Yang et al. 1998; Yoneyama et al. 2015). Se fertilizers have different metabolic specificity among genotypes, and the implications on sugars, fatty acids, and proteins quality were also assessed. It has been reported that biofortification with sodium selenite caused, relatively to sodium selenate, a higher accumulation of Se in the grain (Bashir et al. 2006; Barnett et al. 2010; Bashir et al. 2012; Atique-ur-Rehman et al. 2014; Asad et al. 2015; Imran and Asad 2015). Application of high concentrations of sodium selenite and selenate increased total lipids in all the genotypes, mostly oleic, linoleic, and palmitic acid. It has been noted that concentration pattern being sucrose > glucose > raffinose > fructose and proteins in rice showed enhancement with Se fertilization in rice. Biofortification of crops with Se is more effective with 120–300 g Se ha^{-1}. Micronutrient deficiencies, especially those arising from Se, Zn, and Fe, pose serious human health problems for more than 2 billion people worldwide (Cunningham et al. 1994; Chen et al. 2000; Behrenfeld et al. 2004; Carl et al. 2007; Boonchuay et al. 2013; Das and Green 2013; Imran and Asad 2015). Wheat is a major source of dietary energy and protein for the world's growing population, and

its potential to assist in reducing micronutrient-related malnutrition can be enhanced via integration of agronomic fertilization practices and delivery of genetically manipulated, micronutrient-rich cereal crop (wheat, maize, and rice) varieties. Targeted breeding for these biofortified varieties was initiated by exploiting available genetic diversity for Se, Zn, and Fe from wild relatives of cultivated rice, wheat, and synthetic hexaploid progenitors. The proof-of-concept results from the performance of competitive biofortified wheat lines that showed good adaptation in target environments without compromising essential core agronomic traits. Agronomic biofortification through fertilizer approaches could complement the existing breeding approach; for instance, foliar application of Zn fertilizer can increase grain Zn above the breeding target set by nutritionists (Fan et al. 2001; Fageria et al. 2011, 2012; Fageria 2013; Impa et al. 2013a; Fernando et al. 2014; Imran et al. 2015c). This review synthesizes the progress made in genetic and agronomic biofortification strategies for Zn and Fe enrichment of wheat. Micronutrient malnutrition is of great public health importance in several parts of the world, especially in the developing and underdeveloped countries. It has been estimated that about 2 billion people, about one third of the world's population, are deficient in one or more mineral elements (Graham and Rengel 1993; Food and Board 2001; Foster and Samman 2010; Gao et al. 2011). Although required in traces, these mineral elements are involved in many vital metabolic functions. Micronutrient deficiencies in humans can be remedied through food diversification, mineral supplementation, food fortification, and biofortification (Graham and Welch 1996; Grotz et al. 1998; Graham et al. 2012; Hoekenga 2014; Imran et al. 2015b). Biofortification is the strategy of increasing the content of bioavailable nutrients in the edible parts of staple food crops for better human nutrition. Staple crops such as maize, rice, and wheat provide most of the calories for low-income families around the globe. However, staple crop-based diets fall far short in providing the required amounts of micronutrients, and heavy reliance on staple food is the root cause of micronutrient malnutrition (Hotz and Brown 2004; Högy and Fangmeier 2008; Högy et al. 2009; Impa and Johnson-Beebout 2012; Humayan et al. 2014). Biofortification includes the enhanced uptake of such minerals from soils, their transport to edible plant parts, and improving the bioavailability of these minerals. International initiatives have recently released several plant cultivars with increased bioavailable micronutrient concentrations in their edible parts. The use of these biofortified cultivars is expected to mitigate micronutrient malnourishment in large populations especially in Africa. Crop breeding, genetic manipulation, and application of mineral fertilizers are the bases of biofortification strategies and have enormous potential to address micronutrient malnourishment. In this chapter, crop biofortification for Zn, Fe, vitamin A, and I has been discussed (Impa et al. 2013b; Imran et al. 2015a). Biofortification is a proven strategy to combat micronutrient deficiency in large populations, particularly for those living in developing countries. However, to make it more effective, efficient, and acceptable for people, better planning, implementation, monitoring, and evaluation of biofortification programs are needed to produce cost-effective and socially acceptable biofortified food crops (IPCC 2007; Jocelyn 2007; IRRI 2012). Food safety, quality assurance, and legal framework also need to be considered

while developing any biofortification strategy. Rice and most staple cereals contain low Fe levels, most of which is lost during grain processing. Populations with monotonous diets consisting mainly of cereals are especially prone to Fe deficiency, which affects about two billion people. Supplementation or food fortification programs have not always been successful. Crop Fe fertilization is also not very effective due to Fe soil insolubility (Johnson-Beebout et al. 2009; Johnson et al. 2011; Johnson 2013; Imran et al. 2017; Imran 2018). An alternative solution is Fe biofortification by generating cultivars that efficiently mobilize, uptake, and translocate Fe to the edible parts. Here, we review the strategies used for the Fe biofortification of rice, including conventional breeding and directed genetic modification, which offer the most rapid way to develop Fe-rich rice plants (Katyal and Randhawa 1983; Juliano 1993; Kochian 1993; Koch et al. 1996; Kennedy et al. 2002; Kant et al. 2012; Khan et al. 2015). While classical breeding is able to modify the contents of inhibitors of Fe absorption, transgenic approaches have focused on enhanced Fe uptake from soil, xylem and phloem loading, and grain sink strength (Mandal and Mandal 1986; Marschner 1995, 2012; Krishnaswami 1998; Mäser et al. 2001; Krishnan and Dayanandan 2003; Mabesa et al. 2013). A comprehensive table is provided in which the percentages of the recommended dietary Fe intake reached by independently developed transgenic plants are calculated. In this review we also emphasize that the discovery of new QTLs and genes related to Fe biofortification is extremely important, but interdisciplinary research is needed for future success in this area (McNair et al. 1981; McDonald et al. 2002; Ramesh et al. 2004; McGrath and Lobell 2013; Meng et al. 2014; Myers et al. 2014, 2015; Raliya et al. 2015).

4 Dietary Deficiency in Human Health of Zn, Se, and Fe

Deficiency of Zn is a substantial global public health and nutritional problem. One third of the world population is at risk due to low dietary intake of Zn including 2 billion people in Asia and 400 million in sub-Saharan Africa (Sandström and Lönnerdal 1989; Römheld 1991; Reuter and Robinson 1997; Seneweera and Conroy 1997; Ruel and Bouis 1998; Salunke et al. 2011; Sadeghzadeh 2013; Sasaki et al. 2015). Most of those at risk depend on C_3 grains and legumes as their primary dietary source of Zn and have a high reliance on cereals, especially rice (*Oryza sativa* L.) that has a low Zn concentration with poor bioavailability compared to other cereals (Ramesh et al. 2003; Rebecca 2008; Rayment and Lyons 2010). Therefore, Zn deficiency is a chronic problem among human populations that have rice-based diets.

The low Zn concentration is thought to indirectly result from breeding for high yield and for pest and disease resistance. In addition, modern high-yielding varieties remove large quantities of soil Zn at every harvest, lowering the residual concentration of soil Zn and contributing to lower future grain Zn concentration (Shivay et al. 2008; Shehu and Jamala 2010; Seneweera and Norton 2011; Sharma et al. 2013; Shahzad et al. 2014). Further, the availability of Zn for plant uptake from the soil is affected by the concentrations of macro- and micronutrients, the physico-chemical

and biological properties of a soil, as well as temperature and water availability (Takagi et al. 1984; Suzuki et al. 2008; Takahashi et al. 2009; Shivay et al. 2015; Sundaria et al. 2018). Elevated atmospheric carbon dioxide concentration also reduces the grain micronutrient concentration including Zn. Any genetic and environmental interactions resulting in lower grain Zn concentration in cereals have potentially large negative implications for human health and well-being (Van-Oosten and Besford 1996; Trumbo et al. 2001; Wijesekara et al. 2009; Wang, et al. 2011b, b; Takahashi et al. 2012; Thilakarathne et al. 2014; Verma et al. 2016).

The aim of Zn biofortification of human food grains is to increase Zn concentration and its bioavailability in food, and this appears to be the most feasible, sustainable, and economical approach to address Zn deficiency in the human diet (Yoshida and Tanaka 1969; Zee 1971; Yang et al. 1998; Wolfgang and Bonnie 2007; Wissuwa et al. 2008; Yoneyama et al. 2015). Biofortification could be accomplished genetically through plant breeding and agronomically through Zn fertilization. Identification of the amount of genetic variability for Zn concentration in the germplasm is the initial step, then improving rice Zn concentration. Further, a sound understanding of Zn uptake, root to shoot translocation, distribution, and grain loading is essential to achieve the biofortification target (Takagi et al. 1984; Suzuki et al. 2008; Takahashi et al. 2009).

5 Rice Quality and Factors Effecting Se, Fe, and Zn Uptake

Rice grain Zn concentration is affected by a large number of plant and environmental factors. Plant factors affect the uptake, transport, and remobilization of Zn to developing grains (Johnson-Beebout et al. 2009; Johnson 2013; Imran et al. 2017). The uptake and storage of nutrients are influenced by tissue demand, plant age, and the root system, but all depend on the genetic makeup. Environmental variables that influence the Zn concentration of rice grains include soil Zn status, temperature, and atmospheric (Grotz et al. 1998; Graham et al. 2012; Hoekenga 2014). There is limited understanding of how these plant and environmental factors influence and interact to affect Zn uptake, transport, and loading into the grain. Thus two major questions arise for the development of a rice biofortification program, namely, the extent to which the major determinants of grain Zn concentration are: (1) physiological and genetic mechanisms, or (2) available soil Zn and its management. These propositions are dealt with in detail below.

6 Determinant of Rice Grain Concentration of Zn, Se, and Fe (Fertilizers)

There is increasing evidence that improved growth, yield, and grain Zn concentration could be achieved through Zn fertilization of many crops, including rice (Fan et al. 2001; Fageria et al. 2011, 2012; Fernando et al. 2014; Imran et al. 2015c). Thus, it is important to ensure that there is adequate Zn supply, either by soil Zn

fertilization or foliar Zn application at critical growth stages such as heading and early grain filling (Cunningham et al. 1994; Chen et al. 2000; Carl et al. 2007; Boonchuay et al. 2013; Das and Green 2013).

Nitrogen (N) and phosphorus (P) applications could also considerably influence grain Zn concentration of rice because N application during grain filling promotes Zn uptake and remobilization (Bashir et al. 2006, 2012; Barnett et al. 2010; Atique-ur-Rehman et al. 2014). It has been suggested that synchronizing both Zn and N fertilization might achieve better results than sole application by avoiding the dilution effect. Although high rates of P application may improve shoot growth and grain yield of rice, it may slow Zn uptake by increasing Zn adsorption to soil particles and reducing Zn absorption (Sandström and Lönnerdal 1989; Seneweera and Conroy 1997; Salunke et al. 2011; Sasaki et al. 2015). Most of the Zn used in the field is zinc sulfate fertilizer, which is the most common Zn fertilizer used on rice but which has also been shown to be one of the least effective. It would be useful to investigate how other types of Zn fertilizers improve the Zn bioavailability for the plant. Further, development of improved formulations and delivery methods for Zn application to rice is urgently needed (Mandal and Mandal 1986; Marschner 1995, 2012; Krishnaswami 1998; Mäser et al. 2001; Krishnan and Dayanandan 2003; Mabesa et al. 2013).

On the other hand, dissolved humic substances can complex Zn in soil solution, which can make Zn either less available to plants compared with sorption to cation exchange sites (common in aerobic soils) or more available to plants compared with precipitation of Zn as sulfides or carbonates (common in anaerobic soils) (Shivay et al. 2008; Shehu and Jamala 2010; Seneweera and Norton 2011; Sharma et al. 2013; Shahzad et al. 2014). Higher OM also tends to drive redox potential down faster upon flooding because it provides an additional carbon (C) source for microbial activity, which can cause the low-redox potential precipitation reactions to happen sooner and make Zn less available to rice plants (Van-Oosten and Besford 1996; Trumbo et al. 2001; Wijesekara et al. 2009; Wang et al. 2011a, b; Takahashi et al. 2012; Thilakarathne et al. 2014; Verma et al. 2016). These findings suggest that using Zn fertilizers requires a good understanding of soil conditions, but there is little information on the interaction of genotypes and fertilizer use. Recently, it has been reported that nanoparticles of titanium dioxide and ZnO boost nutrient concentration and growth of tomato plants (Cunningham et al. 1994; Chen et al. 2000; Behrenfeld et al. 2004; Carl et al. 2007; Boonchuay et al. 2013; Das and Green 2013; Imran and Asad 2015). The mechanisms and physiological impact of nanoparticle uptake and translocation should be unraveled. Irrespective of the genotypes used and any differences in Zn efficiency, removal of Zn in grain depletes soil Zn, which must be replaced (Högy and Fangmeier 2008; Hotz and Brown 2004; Högy et al. 2009; Impa and Johnson-Beebout 2012; Humayan et al. 2014).

Zn and Fe concentrations in rice grains relative to other micronutrients, and that the negative effect on Zn may be greater if P is in higher supply (Römheld 1991; Reuter and Robinson 1997; Ruel and Bouis 1998; Sadeghzadeh 2013). These findings emphasize the importance of maintaining soil fertility to improve, or at

least to maintain, existing levels of grain micronutrients, especially Zn and Fe, under e[CO_2].

7 Ways to Overcome Global Dietary Zn, Fe, and Se Deficiency

While it may seem more efficient to directly supplement dietary Zn, this solution is unlikely to be adopted because of cost (Katyal and Randhawa 1983; Juliano 1993; Kochian 1993; Koch et al. 1996; Kennedy et al. 2002; Kant et al. 2012; Khan et al. 2015). Because Zn malnutrition occurs predominantly where poverty is high and accessibility is difficult, those at most risk of deficiency are also those least able to purchase these dietary supplements. A more appropriate strategy is seeking interventions that can raise the concentration of Zn in dietary staples (McNair et al. 1981; McDonald et al. 2002; McGrath and Lobell 2013; Meng et al. 2014; Myers et al. 2015).

To achieve Zn-biofortified grain, greater understanding of the genetic and environmental interactions in controlling Zn homeostasis in rice is urgently needed. The global Zn nutrition goal will require the deployment of a variety of strategies including biofortification by genetic engineering or conventional breeding after screening and genetic analysis of under-utilized rice cultivars, alongside nutrition education and promotion (Yoshida and Tanaka 1969; Zee 1971; Yang et al. 1998; Wolfgang and Bonnie 2007; Wissuwa et al. 2008; Yoneyama et al. 2015). For example, increased Fe concentration of rice endosperm was achieved through overexpression of nicotianamine synthase genes (NAS) or ferritin in conjunction with NAS genes. The single-gene approaches increase Fe concentration twofold and the multi-gene approaches sixfold. Further, it suggested that *OsNAS* genes, particularly *OsNAS2*, have great potential for Fe and Zn biofortification of rice (Graham and Rengel 1993; Food and Board 2001; Foster and Samman 2010; Gao et al. 2011). There is evidence that overexpression of *A. thaliana* Zn transporter in barley (*Hordeum vulgare* L.) doubled the grain Zn concentration, but there are issues with acceptance of genetically modified rice among consumers because of ecological considerations of moving barley genes into the *Oryza* gene pool. Unlike transgenic approaches for biofortification of vitamin A and Fe, it appears that conventional breeding approaches are much more practical in breeding Zn-enriched rice grain (Fan et al. 2001; Fageria et al. 2011, 2012; Fageria 2013; Impa et al. 2013a; Fernando et al. 2014; Imran et al. 2015c).

8 Conclusion

Concentration of micronutrients in rice grains is influenced by plant-related factors and environmental factors and crop management strategies. Improved Zn uptake and efficient remobilization are identified as key bottleneck for Zn, Fe, and Se biofortification. These bottlenecks should be addressed by exploiting the wide genetic diversity of rice germplasm. Micronutrients fertilization will also play an

important role, especially where soils are inherently low in bioavailable nutrients. Consequently, new genetic and management strategies need to be developed to minimize this micronutrient deficiency for people whose staple diet is rice.

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Rice Genetic Engineering for Increased Amino Acid and Vitamin Contents

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Abstract

Plants are the major source of nutrients in the human diet. However, staple cereal crops lack certain amino acids and vitamins, and its nutritious content is not enough to provide a balanced diet. Rice is the most widely preferred food crop; thus, it is necessary that we enhance its nutritional content. This can be achieved through the process of biofortification using principles of genetic engineering. Rice is mainly known to be deficient in threonine and lysine. Hence, there has been great interest in using practical concepts of genetic engineering to increase the amino acid content of rice. Several studies are being carried out on overexpression of aminotransferases in order to obtain significant levels of essential amino acids. Efforts have also been taken to enhance the vitamin content of rice. Golden rice rich in vitamin A is one of the outcomes of such strategies. Similarly, transgenic approaches for the expression of enzymes responsible for synthesis of vitamin E have increased the levels considerably. Thus, genetic engineering and transgenic approaches can prove to be a solution to malnutrition, but further comprehensive study on metabolic pathways and its manipulation is necessary.

Keywords

Amino acids · Biofortification · Genetically modified rice · Lysine · Metabolic engineering · Vitamins

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1 Introduction

Present-day rice varieties were found to be poor in protein content and are deficient in most vital vitamins like A, D, and C. Hence, there is a need to develop protein and vitamin-rich rice genotypes through enhancing the expression of nutritional traits using the different nutrient-rich genetic resources (Tripathy et al. 2017). Agricultural biotechnology has improved the nutrition and production of food crops. Although it has some controversial impact on global economy and environmental safety, it has contributed benefits to agricultural production. Genetically engineered plants have improved crop nutrition as well as yields. Hence, enhancement of proteins, vitamins, and nutrients like iron, zinc, and β-carotene (provitamin A) content in rice can be achieved by using genetic engineering tools (Srivastava 2018).

2 Nutritional Value of Genetically Modified Rice

Rice contains very low quantities of lysine and tryptophan. Hence, there is a need to alter the amino acid composition in seed storage proteins of rice. Amino acid alteration cannot be achieved easily through conventional breeding methods. Molecular techniques may act as powerful tools in this context. Application of single-nucleotide polymorphism (SNP) markers can explain the variations in amino acid composition. Enhancement of rice amino acid through molecular techniques may become a promising solution (Graham et al. 2001). Scientists have detected quantitative trait loci (QTL) for all essential amino acids except tryptophan, glutamine, and asparagine in rice grain (Wang et al. 2008). Some QTLs have been detected on chromosomes 1, 7, and 9, respectively (Zhong et al. 2011). Among these, the QTL cluster related to increased lysine content is present on Chromosome 9 (qAa 9) (Tripathy et al. 2017). The total seed protein content of rice is comprised of 60–80% glutelin controlled by 15 genes and 20–30% prolamin controlled by 34 genes (Kawakatsu et al. 2008; Xu and Messing 2009). Hence, rice protein content (PC) improvement is a major target for biotechnologists. Golden rice includes different genetically modified (GM) rice varieties for enhanced vitamin A content. The first variety of golden rice was developed in 1990 by European scientists. Nearly half of the populations in developing countries are suffering from vitamin A deficiency. This deficiency causes blindness, illness, and even death. Vitamin A deficiency leads to childhood blindness and lowering of immunity. Golden rice differs from common rice as it contains extra genes. These genes are responsible for the enhanced production of provitamin A in the rice grains. Pigmentation of provitamin A-enriched rice grain ranges from yellow to orange, hence the name "golden rice." The golden rice nutritional trait was crossed with popular Asian cultivated rice varieties using conventional breeding methods. These new rice varieties are currently in field trials across Asia. GM rice is anti-allergic and shows potential resistance against lepidopteran insects and tolerance against glufosinate herbicide (Zhang et al. 2016). GM rice contains 35 mg/g of β-carotene. Vitamin A intake strengthens vision, growth, reproduction, cellular differentiation and

proliferation, and integrity of the immune system (Ross and Gardner 1994; Edem 2009; Tang et al. 2009; Huang et al. 2018).

3 Metabolic Engineering of Rice for Improving Essential Amino Acid Contents

Rice is an important source of nutrition and energy for 50% of the world's population, for whom rice has long been a stable diet. The protein content of rice, at approximately 7%, is relatively low compared with that of other cereal grains (Kawakatsu and Takaiwa 2018). Rice protein is a highly digestible and rich source of lysine, leucine, isoleucine, and glycine (Mohan et al. 1988). Out of 20, some amino acids are essential nutritionally, and their deficiency affects nitrogen equilibrium, growth, fertility, and longevity (Mauro and Pietro 1997). In addition, certain amino acids like glycine and glutamic acid appear to be involved in the transmission of impulses in the nervous system (Juan and Salvatore 2006). Inadequate consumption of amino acids than that of recommended dietary allowances decreases hemoglobin levels (Cho et al. 1984; Dwivedi et al. 2012).

Transgenic approach improves the nutritional quality of plant proteins (Sun and Liu 2004). A gene of interest usually from the wild-type encoding desirable trait is identified and isolated for further gene transfer. In plants, a ubiquitous transformation system such as *Agrobacterium* is used, which wound plant tissue to transfer part of its genome with a functional gene into plant cells by integrating with plant genome. This inserted foreign gene is further inherited according to Mendelian concepts (Sautter et al. 2006). Plant metabolic engineering helps in the modification of endogenous pathways, thereby increasing the flux of specific molecules. It helps in the production of more of a specific desired compound, the production of less of a specific unwanted compound, and the production of novel compound(s) (Capell and Christou 2004).

The inability to synthesize certain essential amino acids by animals triggered great interest in increasing these levels in several crop plants. Among these, lysine, tryptophan, and methionine have received utmost attention which are most limited in cereals and legumes (Ufaz and Galili 2008; Galili and Amir 2013; Le et al. 2016). Amino acids represented important targets for metabolic engineering (Galili and Höfgen 2002). Lysine in seeds is synthesized by one of the aspartate family pathways, which is also responsible for synthesis of methionine and threonine. The activity of aspartate kinase (AK) in the lysine biosynthetic pathway in plants is regulated primarily by a lysine-mediated feedback inhibition of dihydrodipicolinate synthase (DHPS) (Galili 1995; Long et al. 2013) (Figs. 1 and 2).

O-phosphohomoserine is the common precursor for biosynthesis of threonine and methionine through threonine synthase (TS) and cystathionine γ-synthase (CGS), respectively, in plants. TS activity is positively regulated by S-adenosylmethionine, a direct product of methionine and flux into threonine synthesis (Galili and Höfgen 2002; Ufaz and Galili 2008). The overexpression of *Arabidopsis* CGS (AtCGS), first intermediate metabolite in methionine pathway, significantly increased both soluble

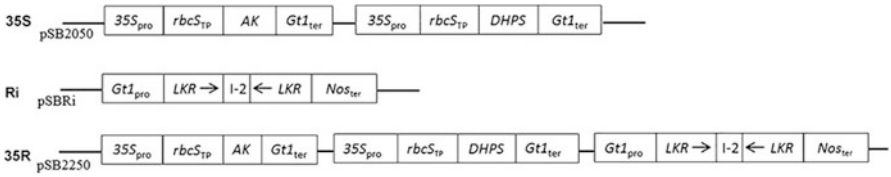

Fig. 1 Transgenic manipulation of lysine metabolism in rice seeds using gene constructs 35S, Ri, and 35R (Reproduced with Permission from Long et al. 2013)

and protein-bound methionine and cysteine in transgenic plants (Avraham et al. 2004). Another important metabolite S-methyl-Met (SMM) is synthesized from Met by Met S-methyltransferase (MMT) for regulating methionine metabolism in plants (Bourgis et al. 1999; Ufaz and Galili 2008). The uptake and reduction of nitrate, incorporation of ammonium into glutamine, and further biosynthesis of glutamate through nitrate reductases, glutamine synthetase (GS), and asparagine synthetase regulate ammonium assimilation. Transgenic modification of GS expressions have been achieved in many plants resulting in plant development, biomass production, and yield (Obara et al. 2001; Miflin and Habash 2002; Gallardo et al. 2003).

Seed proteins of rice are deficient in some essential amino acids. Transgenic rice with increase in content of certain amino acids like glycinin (Katsube et al. 1999a, b), lysine (Lee et al. 2001a, b; Wu et al. 2003), tryptophan (Tozawa et al. 2001), cysteine, and methionine (Hagan et al. 2003; Lee et al. 2003) have been produced in order to provide healthy, balanced, and nutritionally rich diet. Two genes, OASA1 and OASA2, encoding anthranilate synthase (AS), a key enzyme in tryptophan synthesis, α-subunits were isolated from *Oryza sativa* cv Nipponbare (Tozawa et al. 2001; Kawagishi-Kobayashi et al. 2005). The OASA1 transgene proves useful in developing crops with increased Trp content.

Lysine biosynthesis in rice has been enhanced metabolically by overexpressing bacterial lysine feedback-insensitive aspartate kinase (AK) and DHPS through downregulating RNA interference of rice lysine ketoglutaric acid reductase (LKR)/saccharopine dehydropine dehydrogenase (SDH) and by combined expression of AK and DHPS (Long et al. 2013; Liu et al. 2016; Yang et al. 2016). Free lysine was increased in leaves and seeds of transgenic rice (Long et al. 2013). The efficiency of DHPS and bifunctional LKR/SDH in transgenic rice was also published by many authors (Lee et al. 2001a, b; Yang et al. 2016; Wang and Galili 2016). Lysine-rich transgenic rice was developed by Liu et al. (2016) through endosperm-specific expression of foreign lysine-rich protein gene from *Psophocarpus tetragonolobus*.

Escherichia coli serine acetyltransferase isoform (EcSAT) mainly responsible for the synthesis of cysteine precursor O-acetylserine increased both soluble and protein-bound methionine, isoleucine, cysteine, and glutathione in overexpressing EcSAT transgenic rice plants (Nguyen et al. 2012). A chimeric gene encoding sunflower seed albumin, one of the most sulfur-rich seed storage proteins, was introduced into rice in order to modify cysteine and methionine content of seed and total seed protein (Hagan et al. 2003). Sesame 2S albumin is also sulfur-rich seed

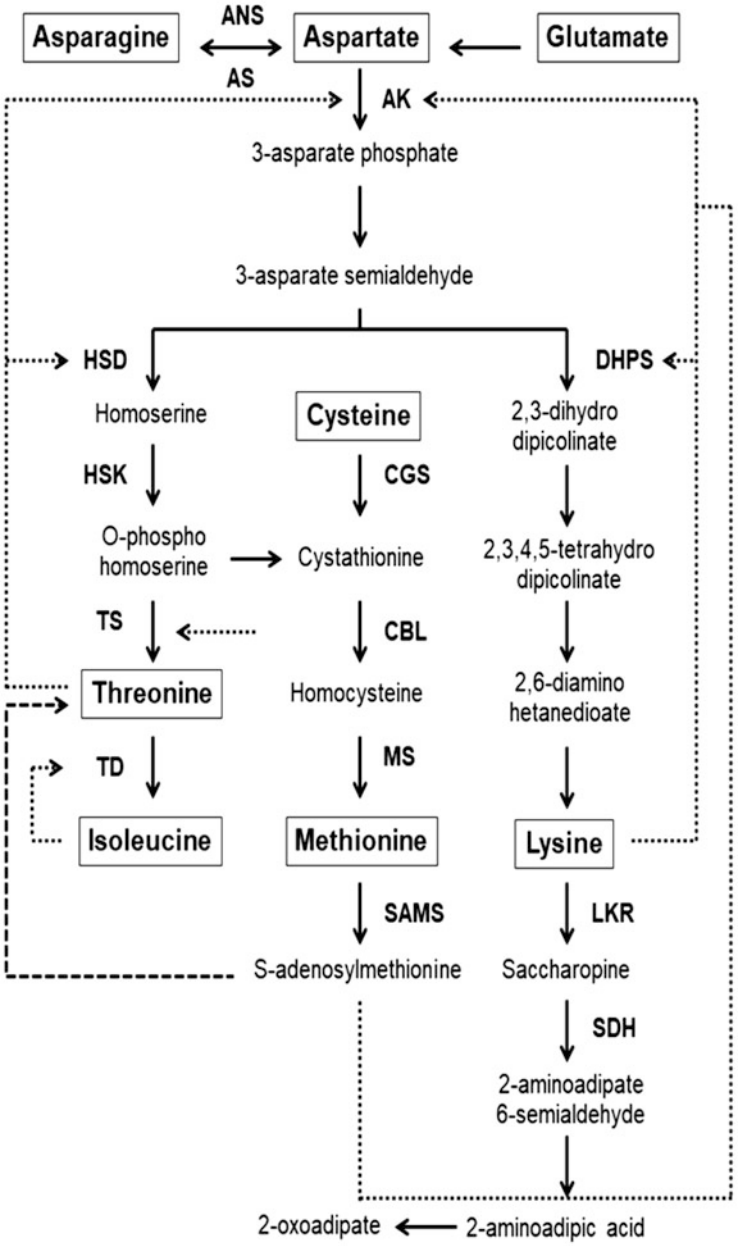

Fig. 2 Biosynthetic pathway of aspartate family for rice genetic engineering to increase amino acid content (*ANS* asparagine synthetase, *AS* anthranilate synthase, *AK* aspartate kinase, *HSD* homoserine dehydrogenase, *DHPS* dihydrodipicolinate synthase, *HSK* homoserine kinase, *CGS* cystathionine γ-synthase, *TS* threonine synthase, *CBL* cystathionine β-lyase, *TD* threonine dehydratase, *MS* methionine synthase, *SAMS* SAM synthase, *LKR* lysine ketoglutaric acid reductase, *SDH* saccharopine dehydropine dehydrogenase) [Reproduced and modified with permission from Galili and Höfgen (2002), Copyrights reserved to Elsevier Inc., 2002 and modified from Long et al. (2013)]

storage protein; it enhanced methionine and cysteine levels in transgenic rice seeds (Lee et al. 2003). The overexpression of aspartate aminotransferase (playing pivotal role in regulating carbon/nitrogen metabolism) gene family of OsAAT from rice and EcAAT from *E. coli* substantially increased amino acid content in seeds of rice (Zhou et al. 2009).

4 Biofortification of Rice for Improved Vitamin Content

Human beings need organic compounds such as vitamins in trace amounts as micronutrients (Jiang et al. 2017b). Due to insufficient intake, vitamins threaten billions of people by causing nutrition-related poor growth (FAO 2009; FAO et al. 2015). Rice feeds more than 50% of the global population and is used as a staple food in many parts of Asia (Bashir et al. 2013). Biofortification or biological fortification is a process of increasing the density of vitamins and minerals in a crop through plant breeding, transgenic techniques, or agronomic practices. Biofortified stable crops, when consumed regularly, will generate measurable improvements in human health and nutrition (Bouis and Saltzman 2017; Garg et al. 2018) (Table 1).

4.1 Vitamins and Enzymes

4.1.1 Vitamin A

Vitamin A is a nutrient of global importance. Shortages in its consumption are estimated to affect 140 million children worldwide. Vitamin A deficiency causes childhood blindness (*xerophthalmia*) in developing countries. Insufficient vitamin A appears prevalence of subclinical deficiency increased by serum retinol level.

Table 1 Biofortification of rice for increased amino acid and vitamin content by transgenic approaches (Reproduced with permission, adapted and modified from Garg et al. 2018)

Nutrients	References
Vitamins	
Beta-carotene	Ye et al. (2000), Beyer et al. (2002), Datta et al. (2003), Paine et al. (2005)
Phytoene	Burkhardt et al. (1997)
Folate	Storozhenko et al. (2007), Blancquaert et al. (2015)
Amino acids	
β-phaseolin	Zheng et al. (1995), Sindhu et al. (1997)
Methionine	Lee et al. (2003)
Cysteine	Lee et al. (2003)
Glycinin	Katsube et al. (1999a, b)
Lysine	Lee et al. (2001a, b), Yang et al. (2016)
Tryptophan	Wakasa et al. (2006)
Aspartic acid	Zhou et al. (2009)

Vitamin A exists in natural products in many different forms. It exists as preformed retinoids, which are stored in animal tissues like the liver and oily fishes, and as provitamin A carotenoids, which are synthesized as yellow pigment by many plants and are found in green, orange, and yellow plants tissues (Combs Jr and McClung 2017). The bioconversion of provitamin A to retinol, the form of vitamin A used by the body, has been found experimentally (Bouis and Saltzman 2017). Gene encoding phytoene synthase (PSY) and phytoene desaturase (PDS) (β-carotene hydroxylase-1 (CRTI)) are widely used for carotenoid biofortification (Jiang et al. 2017b; Garg et al. 2018). The overexpression of 1-deoxy-D-xylulose 5-phosphate synthase leads to increased total carotenoids and β-carotene contents (Giuliano 2017). The β-carotene accumulation is induced by overexpression of the *or* gene product, which enables to induce chromoplast differentiation and carotenoid accumulation (Li and van Eck 2007).

4.1.2 Vitamin B

Vitamin B constitutes water-soluble enzyme cofactors and their derivatives which are essential to various metabolic processes in plants, animals, and microbes (Roje 2007). B_1 (thiamin), B_2 (riboflavin), B_3 (niacin), B_5 (pantothenic acid), B_6 (pyridoxine, pyridoxal, and pyridoxamine), B_8 (biotin), and B_9 (folate) form vitamin B complex (Garg et al. 2018). Folates which are de novo synthesized in bacteria, fungi, and plants play important roles as one-carbon donors/acceptors in almost all organisms (Gorelova et al. 2017; Jiang et al. 2017a, b). Incorporation of vitamin B is very essential in human diet, as human beings cannot synthesize these de novo (Roje 2007). Vitamin B synthesis in plants is consequently as crucial as synthesis, and salvage pathways of certain enzymes and its transports were identified now. Vitamin B synthesis in the *Arabidopsis* and maize is available in public database (SEED). The active form and missing enzymes of B vitamin synthetic pathways were reported earlier; they are vitamin B_1 (thiamin diphosphate: ThDP): phosphatase; Vitamin B_2: phosphatase (PyrP), reductase (PyrR); vitamin B_3: (nicotinamide adenine dinucleotide: NAD and nicotinamide adenine 2'-phosphate: NADP; NMN nucleosidase/adenylyltransferease (nicotinamide mononucleotide:NMN); vitamin B_5: acetohydroxy acid isomeroreductase; vitamin B_6 (pyridoxal 5′-phosphate: PLP, pyridoxamine 5′-phosphate: PMP and pyridoxine 5′-phosphate: PNP): phosphatase; vitamin B_8: 8-amino-7-oxononanoate synthase: KAPAS); vitamin B_9 (tetrahydrofolate:THF)- phosphatase (Gerdes et al. 2012).

4.1.3 Vitamin C

Vitamin C, otherwise known as ascorbate/L-ascorbic acid, can be synthesized through four different pathways (Smirnoff 2011; Jiang et al. 2017a, b). Vitamin C level is quite low (undetectable) in the rice grains against the recommended daily intake of 75 mg per day for adults (Combs Jr and McClung 2017; Jiang et al. 2017b). Overexpression of oxidative stress gene dehydroascorbate reductase through transgenic approaches increased ascorbic acid level which is reported in different plants like rice (Urano et al. 2000), spinach (Shimaoka et al. 2000), wheat (Chen et al. 2003), tomato (Zou et al. 2006), and *Arabidopsis* (Wang et al. 2010). Vitamin C

level in plants increases by overexpression of a gene GalUR that encodes D-galacturonic acid reductase from strawberry. Elevated ascorbate, enhanced growth, and biomass accumulation in *Arabidopsis* were shown in transgenic lines with cloning of myo-inositol oxygenase and $_{L}$-gulono-1,4-lactone oxidase gene by Lisko et al. (2013). AtOxR gene is responsible for tolerance to multiple abiotic stresses and increased vitamin C content in *Arabidopsis* (Bu et al. 2016).

4.1.4 Vitamin E

Vitamin E is usually absorbed and delivered to the liver either in the dietary or supplemental form for human health (Jiang et al. 2017b). Vitamin E supplementation results in decreased risk of cardiovascular disease and cancer, aids in immune function, and prevents or slows several of degenerative diseases associated with aging, such as cataracts, arthritis, and disorders of the nervous system, caused by cumulative damage to tissues mediated by reactive oxygen species (Hirschberg 1999). It is a class of lipid-soluble antioxidants synthesized by photosynthetic organisms (Collakova and DellaPenna 2003). As the prime source of dietary vitamin E, plants produce tocopherol/tocotrienol derivatives which constitute vitamin E (Chen et al. 2006). Seeds of most monocots and limited number of dicots are the major source of vitamin E in the form of tocotrienols. Among these derivatives, deciphering the biosynthetic pathway of α-tocopherol (having highest bioactivity) and the availability of cloned gene for key enzymes has made metabolic engineering of the pathway possible. Increasing the activity of ρ-hydroxyphenylpyruvate dioxygenase (Crowell et al. 2007; Jiang et al. 2017a), homogentisate geranylgeranyl transferase (Yang et al. 2011), homogentisate phytyltransferase (Zhang et al. 2012), tocopherol cyclase (Harish et al. 2013), 2-methyl-6-phytyl-1,4-benzo-quinol methyltransferase (Liu et al. 2008), and γ-tocopherol methyltransferase (Yabuta et al. 2013) is some of the best possible ways to enhance the synthesis of vitamin E (Jiang et al. 2017b).

4.2 Biofortification of Rice

Rice has been the subject in tackling the issues and challenges of food and nutritional security since the past several decades (Garg et al. 2018). Golden rice is a good example of a biofortified crop that contains vitamin A like β-carotene (Combs Jr and McClung 2017). In golden rice, enhanced level of β-carotene is synthesized by the transfer of PSY from the plant daffodil (*Narcissus pseudonarcissus*) and CRT1 PDS from a soil bacterium (*Erwinia uredovora*) (Ye et al. 2000). International agricultural community has taken many efforts to use biofortification breeding techniques. The transfer/overexpression of genes responsible for enzymatic activity in metabolic pathway produces vitamins in rice (Garg et al. 2018).

5 Conclusion and Future Perspectives

The novel application of biotechnological strategies enhances the potentiality of rice production in terms of quality and quantity by improving economically important traits from the species barrier into rice genetic pool. Meanwhile, it can also help in lowering the cost of rice farming and add additional nutritive supplements to rice, thus providing an enriched resource for poor farmers through higher profits and health benefits. The consistent efforts in achieving and implication of biotechnological applications for improvement of rice includes, usage of molecular markers and transgenic technology, to address the concerns targeting on profitability of rice farming such as abiotic stress tolerance, pest resistance, herbicide resistance and value-addition of rice through nutritional enhancement is needed to focus more on sustainable development of future rice cultivars. The main impacts of rice research are ensuring protection of environment, providing food security, tackling climate change, and minimizing poverty. In recent times, significant approaches have been made in gene and genomic insights on protein and amino acids, minerals, vitamin content, phenolic and flavonoid compounds, glycemic index value, and phytic acid content along with QTL analysis, but more research on processing and curative properties are still needed to focus efficiently on achieving optimum benefits. In the post-green revolution era, the enrichment of nutritional value of rice cultivars is very much important in reduction of malnutrition in developing countries. Recently, production of high-protein and zinc-enriched rice varieties address positive note on the progressive step in rice crop improvement program. Hence, transgenic approach will further strengthen to enrich rice nutrition to desired level more effectively. The present knowledge on gene and genomic expression and QTL mapping will provide comprehensive idea in developing desired genotypes. The marker-assisted selection and advancement in biotechniques tools will provide breeders to accumulate and analyze species-specific alleles of genes that play an essential role in the enhancement of nutritional quality in rice varieties.

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Biofortification of Iron, Zinc, and Selenium in Rice for Better Quality

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Abstract

In the last two decades, people are suffering from malnutrition due to the deficiency of certain vitamins and micronutrients in the daily diet worldwide. Micronutrient malnutrition, known as hidden hunger, has been well recognized in the developing world which relayed on cereal-based staple food, particularly in Asian countries, where more than half of the world's population used rice as a staple food. It is well-confirmed that around 60%, 30%, and 15% of the world population are assumed to be deficient in iron (Fe), zinc (Zn), and selenium (Se). As a result, the importance of micronutrient nutrition has to increase at a great pace. In the chapter, we highlight the importance of biofortification in rice and also discuss the agronomic, traditional breeding, biotechnology, molecular, transgenics, and gene editing as potential approaches to combat widespread micronutrient deficiencies in humans.

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1 Introduction

Nutrients are an essential requirement for the growth and development of human beings. We need a number of different elements for our body in whole life, and among them at least 22 mineral nutrients are must for well-being (Samoraj et al. 2018). Micronutrient malnutrition, known as "hidden hunger," was more prevalent in the developing world relayed on cereal-based staple food (FAO 2015). Worldwide people are suffering from malnutrition due to deficiency of those elements in the daily diet. Around 60%, 30%, and 15% of the world population are assumed to be iron (Fe), zinc (Zn), and selenium (Se) deficient (Kassebaum et al. 2014; Vlaic et al. 2019; Wessells et al. 2012; White and Broadley 2009). For instance, Fe is a well-known essential component of hemoglobin and myoglobin, which are involved in oxygen transport and storage. Zinc (Zn) is one of the essential micronutrients, which serves as a co-factor for more than 300 enzymes involved in the metabolism of carbohydrates, lipids, proteins, and nucleic acids, hence its importance in normal growth and development of plants and animals (Roohani et al. 2013). The breeding target to fulfill the 30% estimated average requirement (EAR) of women and children recommended by the HarvestPlus program for Fe is 13 μg/g in polished rice or around five- to sixfold increase of grain Fe in popular rice, while in wheat, it is 59 μg/g (dry weight) of Fe or around twofold (Bouis et al. 2011).

Selenium (Se) deficiency was considered as the fourth most after Fe, Zn, and I (iodine) (White and Broadley 2009) which accounts for approximately one billion entities over the world (Combs 2001). Another study indicated that half of the world population is suffering from Fe, Zn, and Se deficiency (Osendarp et al. 2018). The scenario is quite similar to other micronutrients as well. Nowadays, mineral malnutrition is one of the major global challenges which can be addressed through balanced diet, supplementation, and fortification of food and crops (Kumar et al. 2018). Biofortification (fortification of crops) is one of the solutions that can easily be achieved by applying fertilizers/minerals to the crop plants (Păucean 2017). It became imperative to develop and cultivate varieties that can accumulate more nutrients in the grain and or edible parts from the growing environments. Soil selenium content has a positive correlation in the Se content in foods (Fox and Fairweather-Tait 1999). Food fortification with selenium-containing cooking salt was found to reduce the Keshan disease in China (Cheng and Qian 1990). Selenium can also be fortified in other food items such as yoghurt (Alsuhaibani 2018).

Worldwide research and development in biofortification are mainly focusing on the major/staple crops like rice, wheat, maize, etc. Selenium biofortification is being achieved by using selenium-based fertilizers (Vlaic et al. 2019) and is the safest way to reduce Se deficiency. It becomes bioavailable to humans once it's been assimilated by plants (Lucca et al. 2006). As rice is the predominant food over the

world in more than 30 countries involving 80% daily calorie intake of three billion people around the world (Lucca et al. 2006; Meng et al. 2005), selenium biofortification in rice will have the potential in reducing Se deficiency in a large number of population (Hu et al. 2002). Chen et al. (2002) showed that through fertilization Se content could be increased which will lead to human Se intake.

2 Role in Combating Deficiency

Biofortification, the enhancement of bioavailable micronutrient in the edible parts of staple food by either conventional plant breeding, agronomic approaches, or biotechnology techniques, can help to alleviate malnutrition in the regions where the main source of calories and micronutrients come from staples (Bouis and Saltzman 2017). For biofortification, a better understanding of the key steps of mineral nutrient transport from the rhizosphere to grains is needed, which involves coordination of complex physiological steps such as acquisition of Fe and Zn in roots (uptake), subsequent long-distance transport from roots to shoots, and further redistribution toward the developing seeds (Carvalho and Vasconcelos 2013). Although Fe and Zn are known to accumulate in grains, further insights regarding the underpinning physiology and genetics are yet to be revealed. Soil redox potential and pH affect the uptake of Fe by roots and Zn accumulation in grains. Fe is mostly available in the rhizosphere as low-solubility Fe3 + oxyhydrates, while Fe is oxidized in aerobic soils with high pH, thus occurring as insoluble ferric oxides. Free ferric Fe from the oxides becomes available at low pH for further uptake by roots (Lindsay and Schwab 1982).

Though Se is not an essential element for plants, its beneficial role in growth and developments is already proven (Shanker 2006). Research showed that the beneficial effects of Se can be achieved at low concentrations (Hartikainen et al. 2000; Xue et al. 2001). One of the earliest reports concluded that Se was found as a growth stimulator in mustard (Singh et al. 1980). Since then, several studies suggested that Se has growth-promoting activities in lettuce, ryegrass, soybean, potato, pumpkins, etc. (Djanaguiraman et al. 2005; Germ et al. 2005; Hartikainen and Xue 1999; Hartikainen et al. 1997; Turakainen et al. 2004) while growth rate of seedlings is also promoted by Se (Xue et al. 2001). The growth promotion in plants might be due to the starch accumulation in cell (Pennanen et al. 2002; Xue et al. 2001). Germination of bitter gourd enhanced after seed priming with selenite (Chen and Sung 2001). Delay in senescence was observed in potato and pumpkins (Turakainen et al. 2004; Xue et al. 2001).

3 Uptake and Accumulation in Plants

In nature, the plant acts as a carrier of nutrient elements, carrying from the soil and making ready for human as well as animal consumption. There are so many important factors involved in the middle. Right from uptake to the distribution in

edible product, the major factors are soil nutrient content, nutrient species, bioavailability, plant species, growth stage, soil pH, interaction with other elements, and genes involved in transportations (Koivistoinen 1980; Li et al. 2008; Renkema et al. 2012; Sippola 1979; Ylaranta 1983, 1985; Zhao et al. 2005).

Plants have developed different strategies for Fe uptake from the rhizosphere: Strategy I involving ferrous Fe2+ (non-Poaceae) and Strategy II utilizing ferric Fe3+ (Poaceae), referred to as reducing and chelating strategies, respectively, or a combination of strategies I and II (Connorton et al. 2017). Poaceae family members such as rice (*Oryza sativa* L.), maize (*Zea mays* L.), and wheat (*Triticum aestivum* L.) follow Strategy II, and their root epidermis secretes phytosiderophores (PSs) that form stable Fe (III) chelates in the rhizosphere (Roberts et al. 2004). Soil pH significantly influences Zn acquisition and uptake from the rhizosphere by roots because Zn binds tightly to soil elements and plant cell wall parts under high pH. Details regarding the role and contribution of Zn acquisition by the plant remain unknown. The uptake of Zn may occur as a divalent cation (Zn^{2+}) or as a Zn-PS complex formed with PSs known as Fe^{3+}chelators, which are secreted by the roots of the plant (von Wiren et al. 1996). ZIP-like transporters may take up Zn as noted in Strategy I plants (Ramesh et al. 2003). Plants remobilize and move nutrients from vegetative source organs into seeds during the filling of grains (Waters and Sankaran 2011).

The plants' root absorb Se in the form of selenate and selenite, but the earlier one is the most predominant (Missana et al. 2009; Sors et al. 2005a, b). Accumulation of any molecules to the shoot largely depends on the transpiration rate (Renkema et al. 2012). The translocation of Se from root to shoot happens with the interference of transporters. Selenate and selenite transportation is mediated by phosphate and sulfate transport mechanisms (Feist and Parker 2001; Li et al. 2008; Zhang et al. 2003). In *Arabidopsis*, *SULTR1* and *SULTR1.2* are involved in selenate transportation in plant (El Kassis et al. 2007). Se accumulation generally occurs in young tissues than the older ones (Cappa et al. 2014; Harris et al. 2014) and mostly in vacuoles within plant cells (Mazej et al. 2008). Based on Se accumulation, plants were classified into different groups such as accumulators, indicators, and non-accumulators (Dhillon and Dhillon 2003; Rosenfeld and Beath 1964; Terry et al. 2000; White et al. 2004). Rice can be categorized as non-accumulator, and edible parts contain low Se content (0.029–0.103 mg/kg DM) (Ei et al. 2019), whereas critical concentration in rice plant tissues is 42 μg/g of DW (Rani et al. 2005). The metabolism of Se occurs via sulfur assimilation pathway as it is chemically identical to sulfur (Li et al. 2008; Sors et al. 2005a, b).

Over the last decade, several efforts have been made to biofortify rice with micronutrients, which led to a significant understanding of the genetic and molecular basis of high accumulation in grains and also the influence of agronomic management and environmental factors on micronutrient uptake, translocation, and loading into grains (Impa and Johnson-Beebout 2012). Several genetic studies have also been carried out to identify quantitative trait loci (QTLs) for high Zn in grains, and there is a great potential to use them in marker-assisted breeding.

4 Biofortification Through Multiple Approaches

4.1 Through Agronomic Managements

Agronomic biofortification is a fertilizer-based approach that relies on soil and/or foliar application of micronutrients either alone or in combination with other fertilizers. It is a short-time solution, important to complement the genetic biofortification, particularly when the soil in the target region is limited to a readily available pool of micronutrients (Cakmak and Kutman 2017). For rice, the main challenge is the translocation of the mineral from the vegetative part to the grain (Slamet-Loedin et al. 2015), since it is mostly grown in lowland irrigated areas where Fe is highly available. In contrast with the promising results of foliar fertilizer application to improve grain Zn in rice (Cakmak and Kutman 2017), the increase of grain Fe using foliar fortification was modest. A similar phenomenon was observed in wheat; neither soil nor foliar applications in inorganic form (e.g., FeSO4) or chelated form (e.g., Fe-EDTA, Fe-EDDHA, or Fe-citrate) were reported to be effective for increasing grain Fe concentrations (Cakmak 2008). A minor increase in grain Fe was observed with Fe-EDTA application and also nitrogen application in wheat (Cakmak and Kutman 2017). Another study showed a foliar application of Fe-amino acid (Fe AA) modestly increased grain Fe concentration by 14.5% on average in rice and by 32.5% when 1% (w/v) NA was added (Yuan et al. 2013). A foliar application to reach a significant increase in grain Fe for biofortification remains challenging. It is well-established that a Zn fertilizer strategy is an effective way to biofortify cereal crops with Zn, but the recurrent cost is involved (Cakmak and Kutman 2017). By contrast, genetic biofortification is a seed-based approach that complements agronomic biofortification.

The application method of Se fertilizer is one of the major concerns for biofortification. The method of application should be as such so that plants can easily uptake in its system after application. Application quantity needs to be judicial so efficient uptake and utilization can happen by plants. The excessive amount is normally lost by leaching and impairs the nutrient balance while increasing the production cost (Hirschi 2009; Winkler 2011). Se can be applied to the soil as well as on crops as a foliar spray. Soil enrichment through the application of Se fertilizer to soil could be a component in achieving Se biofortification. Selenate-based fertilizer is found to have potential in enhancing Se assimilation by root and thereby in food products (Ros et al. 2016). Incorporation of Se fertilizer has been practiced in Se-deficient region to fortify the food crops, in Finland (Alfthan et al. 2011), New Zealand (Curtin et al. 2006), China (Wu et al. 2015), the United Kingdom (Hartikainen 2005; Lyons 2010), Africa (Chilimba et al. 2012a, b), and Europe (Poblaciones et al. 2013, 2014). Selenate and selenite are the forms of Se that are generally used, but the earlier one is more common, and those can be applied as granule or powder in wet drench (Shrestha et al. 2006; Broadley et al. 2010; Iwashita and Nishi 2004).

Selenium fortification in rice can be accomplished through foliar application of selenium-containing fertilizer as sodium selenite (Giacosa et al. 2014). The

increasing number of tillers/plant, grain/panicle, and higher grain yields with the spraying of sodium selenite at 10.5–21 g of selenite/ha was obtained by Wang et al. (2013). Protein and lipid content along with selenium content and also grain yield was more in the case of foliar application of selenite alone than other selenium-enriched fertilizers (Hu et al. 2002). Application of selenium-containing fertilizer was found beneficial in increasing selenium content in growing crops (Broadley et al. 2010; Chen et al. 2002; Ríos et al. 2008). A 55-fold increase in rice grain selenium content with the application of 100 g/ha selenite was obtained in a study (Fang et al. 2009). Foliar supplementation of Se was found to increase Se concentration in rice (Boldrin et al. 2013; Pezzarossa et al. 2012). Lyons et al. (2004) claimed the selenium application as the most successful agronomic intervention, to improve the bioavailability of selenium (such as methionine and cysteine). Reports also indicated that an increased amount of rice tissues, particularly in roots, was gained with the application of selenium at 0.5 mg/kg.

4.2 Through the Traditional Breeding Approach

Plant breeding strategies are always a potential option for modifying cultivar with desirable traits. The scope is there for plant breeders to search rice germplasm for sufficient variation in relation to specific traits. Conventional plant breeding has been practiced for centuries to improve the properties of food crops by identifying and developing parent plants with desired characteristics, crossing the parent plants, and selecting offspring with desired agronomic traits inherited from both parent plants (Saltzman et al. 2017). An example of a product developed via plant breeding is high-iron rice variety (IR68144) with high yield, disease tolerance, good tolerance to mineral deficiency, and excellent seed vigor. Furthermore, consumption of IR68144 was reported to have an improvement in iron status of women (Haas et al. 2005). Even though conventional breeding is able to develop high yield and semi-dwarf IR68144, this approach alone in iron biofortification is insufficient in developing a sustainable agronomic plant in terms of yield and quality (Graham et al. 1999).

In 2013, CGIAR-HarvestPlus released a zinc biofortified rice variety developed through conventional breeding in Bangladesh. Currently, about 1.5 million farming households accepted eight varieties of zinc-biofortified rice and have since been growing them (Goldstein 2018). The Indian Institute of Rice Research, Hyderabad, developed a biofortified semi-dwarf, medium-duration (125 days) variety with a non-lodging plant type named IET 23832 (DRR Dhan 45) with a zinc concentration of 22.6–24.00 ppm in polished grain (https://icar.org.in/node/6293, accessed on 14 April 2019). The IET 23832 was also developed by conventional breeding by using HarvestPlus material with some important qualities such as desirable amylose content (20.7%), ensuring good cooking quality, as well as resistance (moderately) to rice blast disease (*Magnaporthe grisea*), sheath rot disease (*Sarocladium oryzae*), and rice tungro virus infection.

Since the uptake and accumulation of micronutrients in edible parts of crops are controlled by polygenes having minor effects, the conventional breeding-based

biofortification approaches have met with only marginal success (Naqvi et al. 2009). Moreover, the success achieved by using this approach depends chiefly on the natural variation that exists in the gene pool. Hence, conventional breeding is best coupled with other approaches such as genetic engineering and agronomic practices to enhance iron content in grains (Jeng et al. 2012; Welch and Graham 2004).

In the case of Se element, efficient absorption, accumulation, and translocation to the reproductive parts could be the essential selection criteria for screening rice germplasm. Research on screening rice germplasm for Se content is not that much, and the existence of genetic variation is not entirely known. Moreover, breeding strategies for improving Se content in grains and edible parts have not been elaborately explained. Numerous attempts have been made to assess variability for Se content in grain for different cereal and legume crops including rice (Norton et al. 2010, 2012; Zhang et al. 2006), durum wheat (Rodríguez et al. 2011), oat (Eurola et al. 2008), barley (Jun et al. 2011), mung bean (Nair et al. 2015), soybean (Yang et al. 2003), several legumes (White 2015), etc.

Appropriate breeding strategies to fortify rice with Se depend on plant interaction to growing environments, soil properties, the bioavailability of Se in soil, Se interaction with other nutrient elements, etc. Selection of germplasm having more Se content in edible products is the prime strategy from breeding point of view. Search for Se interaction with genotypes could yield better cultivar. The hybridization program is an efficient technique for transferring a desirable trait and can be used for Se enrichment as well. Genotype stable performance in multiple testing sites in relation to Se concentration will produce potential ones. Acclimation of biofortified cultivar to a wide range of environment is also essential besides the development (Cakmak 2008). Developing a Se-fortified crop will increase the consumption of this element (White 2015).

4.3 Through Biotechnological Approaches

Modern cultivars have a lower concentration of Fe and Zn in grains than landraces. This is because breeding has been mainly aimed at increasing grain yield or improving host plant resistance, among other target traits, instead of also improving the micronutrient concentration in grain. Utilization of landraces or crop wild relatives for genetic gain is not a new concept. The genetic variability for the content of micronutrients is becoming acknowledged as a desired trait of crop wild relatives, particularly for rice. The wild accessions of rice such as *Oryza rufipogon*, *Oryza nivara*, *Oryza latifolia*, and *Oryza officinalis* seem to be assets in rice improvement, showing higher values for Fe and Zn content than crossbred cultivars (Anuradha et al. 2012).

4.4 Through Molecular Approaches

The finding of quantitative trait loci (QTLs) led to the dissection of complex multigenic traits that were difficult to improve through crossbreeding before the progress made in DNA-aided analysis. QTL mapping for mineral nutrients in grains has allowed the identification of many QTLs for both Fe and Zn (Anuradha et al. 2012; Kumar et al. 2014; Zhang et al. 2011). Most of these QTLs, with a few exceptions, do not seem to be stable across sites. Rice is a model plant for cereal genetics. Chromosome 11 of rice bears a QTL for Zn concentration in the grain, which seems to be associated with OsNAC5—a transcription factor that appears to be related to the remobilization of Zn from green tissues to the seed (Sperotto et al. 2009, 2010). In unpolished rice grains, ten candidate genes known for Fe and Zn homeostasis were localized in the QTL regions, whereas another six candidate genes were close to QTLs on chromosomes 3, 5, and 7, respectively (Anuradha et al. 2012). Based on these results, Anuradha et al. (2012) emphasized the importance of candidate genes OsYSL1 and OsMTP1 for Fe; OsARD2, OsIRT1, OsNAS1, and OsNAS2 for Zn; and OsNAS3, OsNRAMP1, heavy metal ion transport, and APRT for both Fe and Zn biofortification of grain in rice. Recently, Norton et al. (2014) also found several QTLs for grain Zn and other elements in diverse rice genotypes using genome-wide association mapping (GWAS), but the known Zn-related genes were not found in these regions, thereby showing the novelty of their results. Several SSR markers and grain Zn trait associations have also been identified in different populations and germplasm panel of rice (Brara et al. 2015; Zhang et al. 2014). All these tightly linked SNP and SSR markers can be used in MAS. However, there is no literature indicating the successful use of these markers in MAS for improving grain Zn in rice. So, before using these QTLs/genes in MAS, further validation on a large panel of high Zn donor lines and Zn-specific biparental mapping populations is essential. A QTL pyramiding approach with different combinations of these consistent major effect QTLs can be tried in MAS for high grain Zn. As some of these QTLs have large intervals, fine mapping, candidate gene identification, and development of gene-specific markers may facilitate their use in MAS.

Engineering rice plant for an elevated Se uptake and accumulation through molecular tools and strategies has well been reviewed by different authors (Pilon-Smits and LeDuc 2009; Terry et al. 2000). Exploitation of molecular tools and strategies for selenium enrichment in plants has been done by many researchers (Banuelos et al. 2005, 2007; Ellis et al. 2004; LeDuc et al. 2006; Pilon-Smits et al. 1999; Van Huysen et al. 2003; Sors et al. 2005a, b); but only a few efforts were made in rice improvement. Better Se uptake and accumulation were reported in transgenic plants than the non-transgenic one (Pilon-Smits 2012; Pilon-Smits and LeDuc 2009; Terry et al. 2000). An increase in Se content in leaves was reported in *Arabidopsis* by overexpression of the gene ATPS1 (Banuelos et al. 2005; Van Huysen et al. 2003). With the involvement of these particular genes, overexpression of selenocysteine methyltransferase increased the Se concentrations in mustard (Ellis et al. 2004). QTLs have also been detected that influence the Se content in wheat (Pu et al. 2014; Rongzhi et al. 2013), soybean (Ramamurthy et al. 2014), etc. Several QTLs were

found to affect grain Se concentration in the biparental population from indica and japonica (Norton et al. 2010, 2012). Information is gathered from the scientific community related to molecular breeding for Se enrichments day by day and improving our present understanding.

4.5 Through Transgenic Approaches

As a modern weapon to fight against mineral deficiency, genetic engineering to generate transgenics has also been deployed to transfer genes directly into elite genotypes. Transgenic technologies are the tools to improve the genotypes by making changes in focused metabolic pathways. The transgenic strategies to increase the Fe, Zn, and Se content in the crops have been mainly focused on increasing the uptake and utilization efficiency of plants through modulation of transporter expression (Kerkeb et al. 2008).

Studies were carried out to increase the Fe content of the endosperm by expressing ferritin (Kanyshkova et al. 2001). Ferritin, a localized protein in plant plastid, is a major nontoxic stored form of Fe which can release Fe for metabolic functions as and when needed. Being ubiquitous protein, ferritin stores about 4500 Fe atoms in the bioavailable form (Darbani et al. 2002). Therefore, enhancement of Fe accumulation by ferritin gene expression under the control of endosperm-specific promoters is an important strategy for Fe biofortification. Studies found that overexpression of ferritin in several crops increased Fe content as well as bioavailability (Aluru et al. 2011; Qu et al. 2005; Vasconcelos et al. 2003). To increase the Fe accumulation in the endosperm of brown rice seeds, Goto et al. (1999) generated rice transformants of SoyferH1, under endosperm-specific GluB1 rice promoter, and reported a threefold increase in grain Fe content as compared to non-transformed lines.

Several genes/gene families involved in Zn homeostasis have been well characterized in rice. The ZIP family genes are important metal transporters found to be involved in the transport of Zn within and between different parts of the rice plant, and their expression varied with the different Zn conditions (Ishimaru et al. 2007, 2011; Ramesh et al. 2003). Overexpression of OsHMA3 enhanced the uptake of Zn by upregulating the ZIP family genes in the roots (Sasaki et al. 2014). In a study with RILs of Madhukar x Swarna, OsNAS and OsHMA were overexpressed in the leaves (Priya et al. 2015); in the same set of materials under Fe-deficient conditions, NAS2, IRT2, DMAS1, and YSL15 were expressed in the shoot while NAS2, IRT1, IRT2, YSL2, and ZIP8 in the roots (Agarwal et al. 2014). Similarly, Chadha-Mohanty et al. (2015) reported that OsZIP5 and OsFRO1 were upregulated in the roots and flag leaf of high Zn rice lines. Thus, it is very clear that several genes and gene networks are involved in metal uptake, translocation, sequestration, and loading, and their well-coordinated action plays a key role in metal homeostasis in the rice plants.

4.6 Through Gene Editing

A recent development in the genome-editing tool, clustered regularly interspaced short palindromic repeats (CRISPR) for precise modification within the genome, gives researchers a possibility for accurate targeting of genes or genomic regions. This technology has been used in rice to improve yield and stress resistance (Jaganathan et al. 2018; Mishra et al. 2018). The potential example to use CRISPR-based approach is to knockdown OsVIT2 to achieve the increase of grain Fe, similar to the published T-DNA insertion silencing of this gene (Bashir et al. 2013) in different rice cultivars. The development of Fe-enriched rice and wheat grains can also benefit from this method by tweaking the expression of genes involved in Fe homeostasis by editing the regulatory element of Fe homeostasis genes.

5 Implications to Producers and Consumers

Biofortification of the crop with major element becomes popular in many crops. Almost all the studies reported a positive impact of Fe, Zn, and Se application in improving Se content in edible parts. But issue is there whether is it cost-effective for large-scale practice and needed to be a win-win situation for both producer and consumer (Bouis and Welch 2010; Cakmak et al. 2010). Se supplementation sometimes increases biomass or shelf life (Malik et al. 2010; Puccinelli et al. 2017), but yield increase has not been proved. As no additional yield will increase by Se application (Lyons and Cakmak 2012; Ros et al. 2016), price of biofortified rice will be higher than the non-fortified one. However, improvement in edible product quality along with the product price would be commercially worthy to farmers; besides consumers will receive Fe-, Zn-, and Se-enriched products.

6 Summary and Conclusion

From the above discussion of the chapter, it is well-known that people are suffering from malnutrition as a result of the deficiency of vitamins and several micro-elements in the last two decades worldwide, particularly in Asian countries, where more than half of the world's population used rice as a staple food. Among essential micronutrients, Fe, Zn, and Se are the most important since around 60%, 30%, and 15% of the world population have faced the deficiency of these three elements where rice is used as a staple food. To combat widespread micronutrient deficiencies in humans, no alternatives have been recognized without biofortification in rice. However, agronomic, traditional breeding, biotechnology, molecular, transgenics, and gene editing have been found as potential approaches to fighting against micronutrient deficiencies in human health. Among them, both conventional breeding and transgenic approaches have shown that it is possible to increase micronutrient concentrations in staple crops. More than 10 years of transgenic approaches have

revealed useful lessons for future developments of biofortification. For example, it has taught us that increasing the uptake of iron from the soil needs to be combined with increased iron storage capacity. Also, it has been observed that some transgenic strategies increase iron specifically, whereas others (e.g., NAS genes) increase both iron, zinc, and selenium levels. Further gene targets may be identified by forwarding genetics approaches such as QTL mapping or GWAS or gene editing.

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Quantitative Trait Loci for Rice Grain Quality Improvement

Saket Chandra, Aditya Banerjee, and Aryadeep Roychoudhury

Abstract

Rice (*Oryza sativa* L.) is cultivated universally and is one of the most important food crops in the world. However, it lacks many vital vitamins, minerals, fatty acids, essential amino acids, and phytochemicals; therefore further improvement is needed to meet the future needs. New varieties with better yield capability, quality, and resistance to major biotic and abiotic stress will be required to feed the global population. Importantly, enhancement of grain quality has now become the main attention for rice breeding programs because all end users want the best grain quality. The notion of grain quality comprises enhancement in biochemical and physical properties, milling, easiness in cooking, appearance, shape, nutrition, and overall eating quality. Genetic mapping of quantitative trait loci (QTL) controlling grain qualities and genome-wide association studies are implemented to understand the genetic relationship among traits. From the rice reference genome data (*O. sativa* var. *indica* and *O. sativa* var. *japonica*), the search for the candidate genes associated with important traits will be important for molecular-assisted breeding. A number of studies have reported genes and QTLs linked to grain quality, although the trait related to grain quality is intricate. Some genes have been characterized for their role in different biochemical pathways, like starch, flavonoid, protein, and lipid. Molecular tools for marker-assisted selection are available to improve cultivars for better grain quality combining with other important traits. Several recent advances in genomics and QTL studies are enhancing our understanding of molecular mechanisms and pathways that determine genes for quality rice and targeted grain improvement.

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Disease resistance · Abiotic stress · Rice quality · *Oryza sativa*

1 Introduction

Rice (*Oryza sativa* L.) plays a vital role in ensuring food security to majority of the population. Rice is consumed in almost all parts of the world and is the most important staple food crop. Because of its highest consumption as compared to other cereals, it is an important part of the food and source of living of more than 3.5 billion people. Due to the steep increase in the rate of population growth, the world population is about to reach more than 10 billion by the end of the year 2050 (Wing et al. 2018). Moreover, most of the increase in population will take place in underdeveloped and developing countries with high population where consumption of rice is high (like Africa and South Asia), which underlines the importance of rice in this forthcoming threat. Better varieties with good yield potential, high quality, and resistant to major biotic and abiotic stress are the need of the hour to fulfill the demand for the ever-rising population and constant reduction in agricultural land. Enhancing the quality of rice is one of the top priorities for the consumers and plant breeders. Old-style crop improvement program depends on finding and crossing plants with agriculturally important traits. These methods lead to acceptance of semi-dwarf varieties for improved lodging resistance and the utilization of heterosis, which has brought increase in productivity over the past half century (Wing et al. 2018). Nevertheless, this increase in productivity has been expensive in terms of utilization of resources and has cast negative impact on environment in rice-cultivated regions, due to the use of a large amount of fertilizers, pesticides, and a lot of water for irrigation and other resources.

Oryza sativa was believed to be domesticated from the wild grass *Oryza rufipogon* about 14,000 years ago in the Pearl River Valley region in China. *Oryza sativa* is further categorized into two subspecies, namely, *indica* (widespread in tropical regions) and *japonica* (grown mostly in the subtropical and temperate region of East Asia). Both of these subspecies are originated from the same domestication events. One more cultivated species, *O. glaberrima*, which is found in West Africa, is thought to be domesticated much later than *indica* and *japonica* subspecies. *Indica* and *japonica* subspecies share a completely different trait. Development of these two varieties of rice is the result of region-specific preference and preference of certain traits. *Indica* refers to long non-sticky slender grains which are comparatively healthier than *japonica*. On the other hand, *japonica* possesses small, sticky, round, or slender rice grains due to high chalkiness rate. As the name suggests, *japonica* is mainly preferred by Japanese people while *indica* by most of the Asian countries like India, Pakistan, and China.

Quality is termed as "a product with complete feature and appearance or service that provides satisfaction to specified or implied needs" (International Standard Organization (ISO) 8402, 1986). Features are the recognized properties of a product

which are connected with quality standards. Grain quality of rice is one of the important features of rice product that fulfills the need of the consumer. The grain quality comprises numerous features like biochemical properties, physical properties, efficiency of millings, grain shape, size, appearance, instant cooking, taste, and nutrient content. Hence, rice grain quality has been broadly categorized into four types, i.e., milling superiority, grain shape and size, nutritional content, and easiness in cooking and taste. Several countries have established different standards to assess the grain quality. International organizations like ISO (Geneva, Switzerland), Association of Analytical Communities International (AOACI, Maryland, USA), and American Association of Cereal Chemists International (AACCI, Minnesota, USA) have developed protocols to assess few of the quality parameters, for instance, estimation of apparent amylose content. Assessment of quality of grain is not only the key point for the consumers but also for breeders who are responsible for developing new varieties that comprise important quality like better taste, increased productivity, and resistant to biotic and abiotic stress. Market value of rice also primarily depends on grain quality and its appearance, but the parameters deciding the quality differ with geographical region and change with consumer's preference (Fitzgerald et al. 2009). With increase in health awareness among people, nutritional value of rice has also become an important criterion. People want to eat healthy, so the preference of brown rice has increased gradually, and now it is very popular for health-conscious consumers.

To attain this goal, it is important to know well, manage, and exploit the presence of existing genetic variations in domesticated rice and its wild progenitors. For this purpose, comparative and functional studies will serve the purpose to understand and identify the key constituents accountable for agriculturally important traits. Rice is well ahead as compared to other cereals in leading the most recent genomic breeding methods for numerous reasons. Rice is a diploid with a small genome size of approximately 400 Mbp. The genome size of rice is the smallest among cereals, and this feature makes it ideally suited to study genomics of rice with ease. Moreover, rice was the first cereal whose genome is completely sequenced (Matsumoto et al. 2005), and the accessibility of high-quality reference allows functional, population genomic and evolutionary studies. The genus *Oryza* also includes domesticated and wild species with an extended evolutionary path that comprises immense amount of undiscovered reservoir of traits and genes that can be utilized for improvement of crop. Rice has been grown in both the old and new world for many years; during that period, it has adjusted to a variety of geographical locations and different environmental conditions. Rice is now considered as the reference genetic model for other crops (Foley et al. 2011).

Basically, the important traits which need to be considered to develop improved rice variety include grain size and shape, chalkiness, and its color but all uncompromised with its nutritional value and yield. Rice with good quality and quantity can be grown only by exploiting genomics behind its trait. All the traits required to develop a good rice variety including yield are regulated by multiple genes and so are considered as quantitative traits. Therefore, to improve any of these, the associated genes and their sequences, location on a particular chromosome, and

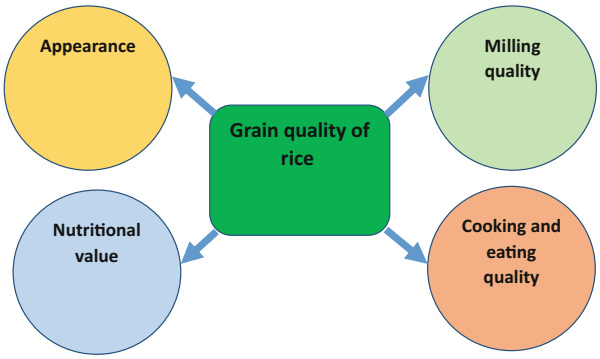

Fig. 1 Factors responsible for determination of rice grain quality

the markers associated with that gene need to be studied. This approach of genomics is more properly known as quantitative trait loci (QTL) analysis. Since rice is the first cereal crop with its fully sequenced genome, it has become a bit easier to perform QTL analysis or genome-wide association studies (GWAS). Sequencing of 517 land races of rice has revealed 18 new genomic loci associated with 10 rice QTLs (Huang et al. 2013).

This chapter focusses on the important genes and existing QTL related to enhancement of grain quality of rice (Fig. 1). The number of QTLs and important genes that have been characterized and fine mapped with their usage in marker-assisted selection will benefit the researchers working on rice for getting a better idea in the genetics and genomics of rice.

2 Properties for Grain Quality

2.1 Milling Quality

This parameter governs the total productivity and the shattered kernel frequency of the milled rice that is of prime importance for growers and consumers. Three important limitations are recovery of white rice (the ratio of white rice to rough rice), recovery of unbroken grains of milled rice (the proportion of unbroken grain of milled rice to rough rice), and recovery of unpolished rice (the proportion of unpolished rice to rough rice). These three parameters play a vital role in determining the competence and superiority of the milling process. Unpolished rice is the rice that is de-hulled with the removal of the two outer bracts (lemma and palea). Unpolished rice is itself a category of complete grain that is used for food. Elimination of outer fiber from unpolished rice that comprises of pericarp, aleurone, embryo, and germ leads to the formation of milled rice. Some polished rice are cracked during the process of milling; the unbroken grains of milled rice are the general term for the complete milled grain. In calculating the unbroken polished grain retrieval, grains lengthier than three-fourth are measured as complete grain.

2.2 Appearance of Grain

It is one of the important assets of rice grain that determine its demand in the market. After milling, the appearance of the grain is linked with a round and long form, transparency, chalkiness, and size. Length of the kernel, thickness, and breadth are the parameters for elaborating the physical measurement of rice grains; the shape of the grain is represented as the proportion of length to width. The length of grain varies from 3 mm to 11 mm, and width ranges from 1.2 mm to 3.8 mm (Fitzgerald et al. 2009; Huang et al. 2013). Generally, the *indica* variety of rice has a slender and long shape, and the *japonica* rice has a round and short grain (Sundaram et al. 2008). Appearance of grain is heavily dependent on the transparency, clearness, and the vitreousness of the endosperm that is often needed in most of the rice industry.

2.3 Quality of Cooking and Eating

This parameter considers the easiness of cooking, stickiness, and firmness of the boiled rice. Cooking of rice and quality of eating is linked to quantifiable physico-chemical properties like consistency of gel, total amylose content, viscosity of pasting, and temperature of gelatinization. All the abovementioned parameters are connected with the property of starch and comprise of 90% of milled rice. Two types of starch are found in rice, namely, amylose and amylopectin. Gelatinization refers to the interruption of arrangement of molecule in the granule of starch established in irreparable fluctuations in property like swelling of granules, melting of native crystallite, solubility of starch, and decrease of birefringence. The temperature of gelatinization identifies the duration and energy needed for cooking. Viscosity of pasting is the parameter that is used to measure the viscosity of rice having similar amylose content. Consistency of gel is the ability of rice to harden on cooling and is generally categorized into soft, medium, and hard.

2.4 Nutritional Value

This parameter is the foremost of all the parameters discussed earlier. Being the key staple food in the world, the quality of nutrition is tightly linked with the health of human and thus is an important criteria for consumers. Carbohydrate and protein are the key constituents of milled rice. The protein and lysine content in rice are the key criteria for determining the nutritional importance of rice. Brown rice is considered as more nutritious than white rice (Shao and Bao 2015).

3 Major QTLs Related to Grain Quality

Advancement of rice grain quality is the major goal for the breeders. Traits for the improvement of rice include appearance, quality of nutrition, milling, and cooking. These trait preferences vary with the region and food habit of the people (Calingacion et al. 2014). Important genes and QTLs for the abovementioned traits are elaborated below.

3.1 QTLs for Quality of Milling

This trait is evaluated based on the recovery of brown rice, head rice, and milled rice. The genetic control behind this trait is very intricate and is not very well understood. Till date, no candidate gene has been genetically recognized and characterized. Nevertheless, numerous reports have been published indicating QTL for the quality of milling (Table 1). These studies expand our knowledge in understanding the genetic regulation of this trait.

About 20 QTLs have been discovered related to brown rice recovery based on previous report. All chromosomes excluding chromosome (chr) 2 comprise of QTL for brown rice recovery. One of the prominent QTLs identified in chr 5 has also the trait related to the control of grain width within the markers C734b and RM42 (Tan et al. 2001). The same QTL region in Chr 3 is also responsible for controlling the grain length (Lou et al. 2009). Both the outcomes support that brown rice recovery is linked with rice kernel size and shape of grain.

For milled rice recovery, there were a total 19 QTLs reported in previous studies, and except chr 8, all chromosome has QTL for milled rice recovery. All the QTLs reported for this trait has fractional effect. Three independent experiments identified QTLs for the recovery of milled rice on chr 5 (Tan et al. 2001; Aluko et al. 2004; Zheng et al. 2007), although all three are located in different genomic regions.

For the head rice recovery, about 34 QTLs have been identified in ten studies with the number of QTLs ranging from one to seven in independent experiments. An important QTL identified in chr 3 for head rice recovery and also another trait controlling for grain length have been found in the same region (Tan et al. 2001), indicating that both the traits are connected to each other. Other studies have also reported the QTL for head rice recovery in chr 3, suggesting that there is an important gene for head rice recovery located in this chr (Li et al. 2004; Dong et al. 2004; Aluko et al. 2004; Jiang et al. 2005; Lou et al. 2009). Moreover, in chr 1, 5, and 6, QTL has been identified for head rice recovery in three different studies. This trait is also affected by the abiotic factors especially in environmental interactions. There is strong correlation for the recovery of head rice with early heading QTLs in the warm parts of the world, indicating that environment also plays an important role in the expression of this QTL.

Table 1 Important QTLs reported for traits related to milling quality

Parental population	Characteristics	Chromosome (Chr)	References
Minghui 63/Zhensan 97	Recovery of brown rice	Chr 5	Tan et al. (2001)
Kasalath/ Nipponbare	Recovery of brown rice	Chr 3, Chr 4, Chr 9, Chr 10, Chr 11	Li et al. (2004)
IR-24/Asominori	Recovery of brown rice	Chr 9, Chr 10	Dong et al. (2004)
O. glaberrima/ Caiapo	Recovery of brown rice	Chr 1, Chr 7, Chr 8	Aluko et al. (2004)
WYJ-2/Zhensan 97	Recovery of brown rice	Chr 12	Jiang et al. (2005)
Lemont/Teqing	Recovery of brown rice	Chr 5, Chr 6, Chr 7	Zheng et al. (2007)
Nanyangzhan/ Chuan7	Recovery of brown rice	Chr 1, Chr 3	Lou et al. (2009)
01Y110/L204	Recovery of brown rice	Chr 1, Chr 4, Chr 6	Nelson et al. (2012)
Minghui 63/Zhensan 97	Recovery of milled rice	Chr 3, Chr 5	Tan et al. (2001)
Kasalath/ Nipponbare	Recovery of milled rice	Chr 4, Chr 9, Chr 10, Chr 11	Li et al. (2004)
IR-24/Asominori	Recovery of milled rice	Chr 11, Chr 12	Dong et al. (2004)
O. glabberrima/ Caiapo	Recovery of milled rice	Chr 5, Chr 7	Aluko et al. (2004)
Lemont/Teqing	Recovery of milled rice	Chr 1, Chr 2, Chr 5, Chr 6, Chr 7	Zheng et al. (2007)
Nanyangzhan/ Chuan 7	Recovery of milled rice	Chr 3	Lou et al. (2009)
01Y110/ L204	Recovery of milled rice	Chr 1, Chr 4, Chr 9	Nelson et al. (2012)
Minghui 63/Zhensan 97	Recovery of head rice	Chr 3	Tan et al. (2001)
O. rufipogon/ IR-64	Recovery of head rice	Chr 1, Chr 2, Chr 5	Septiningsih et al. (2003)
Kasalath/ Nipponbare	Recovery of head rice	Chr 3, Chr 6, Chr 7	Li et al. (2004)
IR-24/Asominori	Recovery of head rice	Chr 1, Chr 3, Chr 5	Dong et al. (2004)
O. glaberrima/ Caiapo	Recovery of head rice	Chr 1, Chr 3, Chr 6, Chr 8, Chr 11	Aluko et al. (2004)
WYJ-2/Zhensan 97	Recovery of head rice	Chr 3, Chr 8	Jiang et al. (2005)
Lemont/Teqing	Recovery of head rice	Chr 1, Chr 5, Chr 6	Zheng et al. (2007)

(continued)

Table 1 (continued)

Parental population	Characteristics	Chromosome (Chr)	References
Nanyangzhan/ Chuan 7	Recovery of head rice	Chr 3	Lou et al. (2009)
RT0034/cypress	Recovery of head rice	Chr 6, Chr 9	Nelson et al. (2011)
LaGrue/ cypress	Recovery of head rice	Chr 1, Chr 5, Chr 9, Chr 10	Nelson et al. (2011)
01Y110/L204	Recovery of head rice	Chr 6, Chr 8, Chr 9, Chr 10, Chr 11	Nelson et al. (2012)

3.2 QTLs for Rice Grain Appearance

Traits related to grain size, like width, thickness, and length of grain and length to width ratio play a major role in enhancing the grain quality and weight and leading to the release of new varieties (Fitzgerald et al. 2009; Huang et al. 2013; Zuo and Li 2014). It is well-established fact that productivity of rice depends on weight of grain, number of panicles, and number of grains per panicle. Of all these three factors, weight of the grain is the main factor. Hence, increasing the weight of grain by expanding the size of grain will be an important step to enhance the productivity. The main grain appearance qualities have been discussed below:

(a) **Grain shape and size**: Shape of the grain is the important factor for determining the grain quality that is also connected with yield potential of grain. A slender and long grain of rice is mostly liked by residents of South China, USA, and South and Southeast Asia; on the other hand, residents of Korea, Northern China, and Japan prefer round and short grains of rice (Huang et al. 2013). Width, length, and shape of grain are the important and constant qualities of any cultivar, so that they are transferable to the next generation of crops. In terms of heredity, a number of QTLs have been discovered for length, width, and shape of grain. Chromosome 3 has been reported to have the maximum number of QTL. The fine-mapped QTL imparts probable markers for molecular breeding to manipulate shape of grain with the implementation of molecular markers resulting from the cloned genes, and this will help in exact trait phenotype in breeding. Mapping of QTL indicates that numerous QTLs portray pleiotropic effects; they regulate not only the length of grain but also width of the grain, productivity, and shape of grain (Fan et al. 2006; Song et al. 2007; Guo et al. 2009; Bai et al. 2010; Li et al. 2011; Wang et al. 2012). Characterization of cloned genes imparts better resources for understanding the pleiotropic effect (Fan et al. 2006; Song et al. 2007; Li et al. 2011; Wang et al. 2012).

(b) **Grain length:** The length of rice grains is an important quality associated with high market value and demand. QTL analysis of F2 and F3 derived lines generated from the cross between the long grain *japonica* cultivar Cytoto and

the *indica* cultivar Kasalath led to the identification of three QTLs for grain length. These QTLs were detected on chr 3, 4, and 7 (Kato et al. 2011). The QTL detected on chromosome 4 was reported as *qGL4b* and was associated with increased length and weight of rice grain (Kato et al. 2011). Segami et al. (2016) detected 12 loci which contained 14 QTLs associated with grain length. The effects of *GL3b* and *GL6* on rice grain length were confirmed by progeny testing (Segami et al. 2016). Yu et al. (2017) used GWAS to identify 99 QTLs associated with grain length and ten million single nucleotide polymorphisms (SNPs) in 504 rice accessions. Linkage-based association mapping was used to detect novel genes related to grain length. Cloning of one of the new genes, i.e., *OsLG3*, showed that the gene positively regulated grain length in rice (Yu et al. 2017).

(c) **Rice grain chalkiness and color**: Grain chalkiness reduces market value of rice grain. Grains with high chalkiness are very low in amylose content and impart waxy texture to the cooked grain. It is a typical trait which is substantially influenced by environmental factors. In general, high temperature enhances chalkiness. There are not much QTL which has been identified for this trait. *Chalk5* located at chr 5 is the major QTL identified for this trait which positively regulates white belly rates in grain.

Apparent amylose content (AAC) was investigated while studying genetic factors involved in regulating grain quality (Bao et al. 2002). Other characteristics measured to study QTL related with grain quality were gelatinization temperature (GT), gel consistency (GC), and six starch pasting viscosity parameters. This involved using 193 molecular markers mapped on a doubled haploid (DH) population consisting of 135 lines. This led to the discovery of 17 QTLs, none in the *wx* locus, but in total connected with nine traits. Polygene-mediated control and regulation of grain shape and chalkiness have been reported. These traits were found to be hereditary and uninfluenced by abiotic factors (Hofmann 2012).

4 Conclusion and Future Perspectives

Rice is the major cereal staple food crop across the world. Production of high-quality rice grains with superior flavor is of high demand in national and international markets. Scientists and breeders have adopted genomic and QTL-assisted approaches to study major traits associated with superior rice varieties. Identification of novel QTLs and SNPs can aid in understanding the molecular basis of a particular trait in the grain which will lead to the incorporation of the superior trait in inferior varieties. This would enhance rice production and the quality of rice grains throughout the world. QTL-dependent artificial screening and selection of mega yielding rice varieties would obviously aid in feeding the growing population across the world.

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Improvement of Rice Quality via Biofortification of Selenium, Iron, and Zinc and Its Starring Role in Human Health

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Abstract

The Consultative Group for International Agricultural Research (CGIAR) aims to support all efforts to improve the nutritional value of rice—in particular iron, zinc, selenium, vitamin A, calcium, and iodine, which are usually low in rice—particularly in rice-consuming communities. Rice biofortification with micronutrient is the only tool for reducing micronutrient malnutrition in staple foods whose edible portions in bioavailable minerals and vitamins are denser. Cereals are often the most productive but are generally low in micronutrients. In developing countries, cereals dominate the dietary system and thus appear to be the most likely reason of micronutrient deficiencies in the society. The aromatic cultivars have consistently higher concentration of iron and zinc in grain than the nonaromatic types. Zinc has multiple roles in the human body including the

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efficient functioning of cellular metabolic activities and stimulation of the immune system. Selenium is an essential element for human health but its intake is low. Accordingly, biofortified rice with this trace element can be prophylactic to consumers. Micronutrient deficiencies, especially those arising from selenium (Se), zinc (Zn), and iron (Fe), pose serious human health problems for more than 2 billion people worldwide.

Keywords

Rice (*Oryza sativa* L.) micronutrients · Zinc · Selenium · Iron · Biofortification

1 Introduction

Rice is the world's largest and most important staple food among cereal crops. Productivity, quality and profitability of rice have become an integral part of the nutritional food system (Krishnaswami 1998; Mabesa et al. 2013; Mandal and Mandal 1986; Marschner 1995, 2012; Mäser et al. 2001). Micronutrient malnutrition in rice is a common phenomenon due to deficiency of selenium, iron, zinc, iodine and vitamin A and may cause lower resistance to diseases in children and reduced the probability of child survival at birth (Shahzad et al. 2014; Sharma et al. 2013; Shehu and Jamala 2010; Shivay et al. 2008; Takahashi et al. 2012; Thilakarathne et al. 2014). Micronutrient concentration in grain can effectively be enhanced by application of appropriate mineral forms. The sources of micronutrients are inorganic, synthetic chelates or natural organic complexes (Boonchuay et al. 2013; Carl et al. 2007; Chen et al. 2000; Cunningham et al. 1994; Das and Green 2013; Imran and Khan 2015; Högy and Fangmeier 2008; Högy et al. 2009). It has been reported that the magnitude of yield response as well as Zn, Se, and Fe uptake by rice was enhanced with application of compost. Organic amendments, especially FYM, increase the concentrations of many nutrients and can enhance the nutritional value and nutrient balance of plant foods (Römheld 1991; Ruel and Bouis 1998; Sadeghzadeh 2013; Juliano 1993; Kant et al. 2012; Katyal and Randhawa 1983; Kennedy et al. 2002; Khan et al. 2015a, b; Koch et al. 1996; Kochian 1993; McDonald et al. 2002; McGrath and Lobell 2013; McNair et al. 1981). Organic acids such as citric, malic, oxalic, and phenolic that form Fe complexes are released when organic matter decomposes. In human nutrition terms, necessary for human health, bioavailability is commonly defined as the amount of a nutrient in a meal that is absorbable and utilizable by the person eating the meal (Graham et al. 2012; Grotz et al. 1998; Hoekenga 2014; Imran et al. 2015a; Wissuwa et al. 2008; Wolfgang and Bonnie 2007). The total amount of a micronutrient in a plant food does not represent the actual micronutrient content of the food that is utilizable by the consumer. This quantity must be determined independently using methodologies especially developed for such purposes. Micronutrient malnutrition is a leading health-care issue in the world today and much more detectable in developing countries (Foster and Samman 2010; Gao et al. 2011; Graham and Rengel 1993; Myers et al. 2014; Raliya et al. 2015). Selection of nutritionally rich (aromatic) varieties of rice can form the

basis for a food-based solution to the nutritional needs of the population. Micronutrient malnutrition is of great public health importance in several parts of the world, especially in developing and underdeveloped countries (Fageria et al. 2011, 2012; Imran et al. 2015b; Fan et al. 2001; Fernando et al. 2014; Impa et al. 2013a). It has been estimated that about 2 billion people, about one third of the world's population, are deficient in one or more mineral elements. Although required in traces, these mineral elements are involved in many vital metabolic functions. Micronutrient deficiencies in humans can be remedied through food diversification, mineral supplementation, food fortification, and biofortification (Imran et al. 2017; Johnson 2013; Johnson-Beebout et al. 2009; Imran 2018; Shivay et al. 2015; Sundaria et al. 2018). Biofortification is the strategy of increasing the content of bioavailable nutrients in the edible parts of staple food crops for better human nutrition (IRRI 2012). Staple crops such as maize, rice, and wheat provide most of the calories for low-income families around the globe. Micronutrient deficiencies, especially those arising from selenium (Se), zinc (Zn), and iron (Fe), pose serious human health problems for more than 2 billion people worldwide (Johnson et al. 2011; Imran et al. 2017; Johnson 2013; Johnson-Beebout et al. 2009; Imran 2018; Shivay et al. 2015). Biofortification is a proven strategy to combat micronutrient deficiency in large populations, particularly for those living in developing countries. However, to make it more effective, efficient, and acceptable for people, better planning, implementation, monitoring, and evaluation of biofortification programs are needed to produce cost-effective and socially acceptable biofortified food crops (Yang et al. 1998; Yoneyama et al. 2015). Food safety, quality assurance, and legal framework also need to be considered while developing any biofortification strategy.

2 Starring Role of Se, Fe, and Zn in Human Health

Application of high concentrations of sodium selenite and selenate increased total lipids in all the genotypes, mostly oleic acid, linoleic, and palmitic acid. It has been noted that the concentration pattern of sucrose > glucose > raffinose > fructose and protein in rice has been shown to improve with selenium fertilization in rice. Biofortification of selenium crops is more effective at 120–300 g Se ha^{-1}. Selenium fertilizers have different metabolic characteristics between genotypes, and the effects on sugars, fatty acids and protein quality have also been assessed. Biofortification with sodium selenite has been reported to have caused a higher accumulation of selenium in the grain relative to sodium selenate. Selenium is an essential component of human health, but its intake is low. (Atique-ur-Rehman et al. 2014; Barnett et al. 2010; Bashir et al. 2006; Bashir et al. 2012; Imran and Khan 2015; Yang et al. 1998). Micronutrient deficiencies, especially those arising from selenium (Se), zinc (Zn), and iron (Fe), pose serious human health problems for more than 2 billion people worldwide (Behrenfeld et al. 2004; Boonchuay et al. 2013; Carl et al. 2007; Chen et al. 2000; Cunningham et al. 1994; Das and Green 2013; Imran and Khan 2015). Wheat is a major source of dietary energy and protein for the world's growing population, and its potential to assist in reducing micronutrient-related malnutrition

can be enhanced via integration of agronomic fertilization practices and delivery of genetically manipulated, micronutrient-rich cereal crop (wheat, maize, and rice) varieties. Targeted breeding for these biofortified varieties was initiated by exploiting available genetic diversity for Se, Zn, and Fe from wild relatives of cultivated rice, wheat, and synthetic hexaploid progenitors. The proof-of-concept results from the performance of competitive biofortified wheat lines showed good adaptation in target environments without compromising essential core agronomic traits. Agronomic biofortification through fertilizer approaches could complement the existing breeding approach; for instance, foliar application of Zn fertilizer can increase grain Zn above the breeding target set by nutritionists (Fageria 2013; Fageria et al. 2011, 2012; Imran et al. 2015b; Fan et al. 2001; Fernando et al. 2014; Impa et al. 2013a). This review synthesizes the progress made in genetic and agronomic biofortification strategies for Zn and Fe enrichment of wheat. Micronutrient malnutrition is of great public health concern in several parts of the world, especially in developing and underdeveloped countries. It has been estimated that about 2 billion people, about one third of the world's population, are deficient in one or more mineral elements (Food and Board 2001; Foster and Samman 2010; Gao et al. 2011; Graham and Rengel 1993). Although required in traces, these mineral elements are involved in many vital metabolic functions. Micronutrient deficiencies in humans can be remedied through food diversification, mineral supplementation, food fortification, and biofortification (Graham and Welch 1996; Graham et al. 2012; Grotz et al. 1998; Hoekenga 2014; Imran et al. 2015a). Biofortification is the strategy of increasing the content of bioavailable nutrients in the edible parts of staple food crops for better human nutrition. Staple crops such as maize, rice, and wheat provide most of the calories for low-income families around the globe. However, staple crop-based diets fall far short in providing the required amounts of micronutrients, and heavy reliance on staple food is the root cause of micronutrient malnutrition (Högy and Fangmeier 2008; Högy et al. 2009; Hotz and Brown 2004; Humayan Kabir et al. 2014; Impa and Johnson-Beebout 2012). Biofortification includes the enhanced uptake of such minerals from soils, their transport to edible plant parts, and improved bioavailability of these minerals. International initiatives have recently released several plant cultivars with increased bioavailable micronutrient concentrations in their edible parts. The use of these biofortified cultivars is expected to mitigate micronutrient malnourishment in large populations especially in Africa. Crop breeding, genetic manipulation, and application of mineral fertilizers are the bases of biofortification strategies and have enormous potential to address micronutrient malnourishment. In this chapter, crop biofortification for zinc, iron, vitamin A, and iodine has been discussed (Impa et al. 2013b; Imran et al. 2015a, b, c). Biofortification is a proven strategy to combat micronutrient deficiency in large populations, particularly for those living in developing countries. However, to make it more effective, efficient, and acceptable for people, better planning, implementation, monitoring, and evaluation of biofortification programs are needed to produce cost-effective and socially acceptable biofortified food crops (IRRI 2012; IPCC 2007; Zuckerman 2007). Food safety, quality assurance, and legal framework also need to be considered while developing any biofortification strategy. Rice, the most

staple cereal, contains low iron (Fe) levels, most of which is lost during grain processing. Populations with monotonous diets consisting mainly of cereals are especially prone to Fe deficiency, which affects about two billion people. Supplementation or food fortification programs have not always been successful. Crop Fe fertilization is also not very effective due to Fe soil insolubility (Johnson et al. 2011; Imran et al. 2017; Johnson 2013; Johnson-Beebout et al. 2009; Imran 2018). An alternative solution is Fe biofortification by generating cultivars that efficiently mobilize, uptake, and translocate Fe to the edible parts. Here, we review the strategies used for the Fe biofortification of rice, including conventional breeding and directed genetic modification, which offer the most rapid way to develop Fe-rich rice plants (Juliano 1993; Kant et al. 2012; Katyal and Randhawa 1983; Kennedy et al. 2002; Khan et al. 2015a, b; Koch et al. 1996; Kochian 1993). While classical breeding is able to modify the contents of inhibitors of Fe absorption, transgenic approaches have focused on enhanced Fe uptake from soil, xylem, and phloem loading and grain sink strength (Krishnan and Dayanandan 2003; Krishnaswami 1998; Mabesa et al. 2013; Mandal and Mandal 1986; Marschner 1995, 2012; Mäser et al. 2001). A comprehensive table is provided in which the percentages of the recommended dietary Fe intake reached by independently developed transgenic plants are calculated. In this review, we also emphasize that the discovery of new QTLs and genes related to Fe biofortification is extremely important, but interdisciplinary research is needed for future success in this area (McDonald et al. 2002; McGrath and Lobell 2013; McNair et al. 1981; Meng et al. 2014; Myers et al. 2015, 2014; Raliya et al. 2015; Ramesh et al. 2004).

3 Insufficiency of Zn, Se, and Fe in Human Health

Low concentration of Zn is thought to result indirectly from high yield breeding and pest and disease resistance. In addition, modern high-yielding varieties remove large quantities of soil Zn at each harvest, reduce the residual concentration of soil Zn, and contribute to a lower future concentration of grain Zn (Seneweera and Norton 2011; Shahzad et al. 2014; Sharma et al. 2013; Shehu and Jamala 2010; Shivay et al. 2008). Zinc (Zn) dietary deficiency is a major global public health and nutrition problem. One-third of the world's population is at risk due to low dietary intake of Zn, including 2 billion and 400 million people in Asia and sub-Saharan Africa, respectively (Reuter and Robinson 1997; Römheld 1991; Ruel and Bouis 1998; Sadeghzadeh 2013; Salunke et al. 2011; Sandström and Lönnerdal 1989; Sasaki et al. 2015; Seneweera and Conroy 1997). Most of those at risk depend on C_3 grains and legumes as their primary dietary source of Zn and have a high reliance on cereals, especially rice (*Oryza sativa* L.) that has a low Zn concentration with poor bioavailability compared to other cereals (Ramesh et al. 2003; Rayment and Lyons 2010; Rebecca 2008). Therefore, Zn deficiency is a chronic problem among human populations that have rice-based diets.

Further, the availability of Zn for plant uptake from the soil is affected by the concentrations of macro- and micronutrients, the physicochemical and biological

properties of a soil, as well as temperature and water availability (Shivay et al. 2015; Sundaria et al. 2018; Suzuki et al. 2008; Takagi et al. 1984; Takahashi et al. 2009). Elevated atmospheric carbon dioxide concentration also reduces the grain micronutrient concentration including Zn. However, in wheat, future-induced deleterious effects on grain mineral composition may be complicated by rising temperatures and increased water deficits. Any genetic and environmental interactions resulting in lower grain Zn concentration in cereals have potentially large negative implications for human health and well-being (Takahashi et al. 2012; Thilakarathne et al. 2014; Trumbo et al. 2001; Van Oosten and Besford 1996; Verma et al. 2016; Wang et al. 2011a, b; Wijesekara et al. 2009).

The aim of Zn biofortification of human food grains is to increase Zn concentration and its bioavailability in food, and this appears to be the most feasible, sustainable, and economical approach to address Zn deficiency in the human diet (Wissuwa et al. 2008; Wolfgang and Bonnie 2007; Yang et al. 1998; Yoneyama et al. 2015; Yoshida and Tanaka 1969; Zee 1971). Biofortification could be accomplished genetically through plant breeding and agronomically through Zn fertilization. Identification of the amount of genetic variability for Zn concentration in the germplasm is the initial step and then improving rice Zn concentration. Further, a sound understanding of Zn uptake, root to shoot translocation, distribution, and grain loading is essential to achieve the biofortification target (Suzuki et al. 2008; Takagi et al. 1984; Takahashi et al. 2009).

4 Malnutrition Is a Global Perspective

Zinc deficiency is recognized as one of the major nutrient disorders in humans and has more profound effects in children (McGrath and Lobell 2013; McNair et al. 1981; Meng et al. 2014; Myers et al. 2015, 2014; Raliya et al. 2015). Zinc has multiple roles in the human body including the efficient functioning of cellular metabolic activities and stimulation of the immune system (Thilakarathne et al. 2014; Trumbo et al. 2001; Van Oosten and Besford 1996; Verma et al. 2016; Wang et al. 2011a). Zinc is also present in nearly 300 enzymes in the human body and is important for bone mineralization; the growth of body tissues and the fetus; sperm production and fertility; smell, vision, taste, and appetite; healthy growth of the skin, hair, and nails; as well as blood clotting and wound healing, functioning of the immune system and thyroid, cell division, and protein and DNA synthesis (Römheld 1991; Ruel and Bouis 1998; Sadeghzadeh 2013; Salunke et al. 2011; Sandström and Lönnerdal 1989). Daily intake of Zn is important as the mammalian body has limited Zn stores, and the daily requirement is influenced by gender and physiological stage.

Zinc deficiency is responsible for the development of a large number of illnesses and diseases including stunting of growth, compromised immune system function, cancer, susceptibility to infectious diseases, iron deficiency anemia, and poor birth outcomes in pregnant women, hair and memory loss, skin problems, weakening of body muscles, infertility in men, and pneumonia in children (Krishnaswami 1998; Mabesa et al. 2013; Mandal and Mandal 1986; Marschner 1995). Impaired Zn

homeostasis is associated with several diseases, including diabetes mellitus and zincuria which is one of the symptoms of diabetes. Zinc supplementation amends glycemia in both type 1 and type 2 diabetes. Zinc can be supplemented through dietary sources such as seafood, meat, green leafy vegetables, and grains (Katyal and Randhawa 1983; Kennedy et al. 2002; Khan et al. 2015a, b; Koch et al. 1996). However, maintaining a sufficient Zn concentration in rice grain is important for more than half of the world's population for whom rice is the staple diet.

5 Factors Affecting Se, Fe, and Zn Uptake in Rice

The concentration of Rice grain Zn is affected by a large number of plant and environmental factors. Plant factors have an impact on the uptake, transport and remobilization of Zn for grain development (Imran et al. 2017; Johnson 2013; Johnson-Beebout et al. 2009). The uptake and storage of nutrients is influenced by the demand for tissue, plant age, and root system, but all of them depend on genetic makeup. Environmental variables that influence the concentration of rice grains in Zn include soil status, temperature, and atmospheric status (Graham et al. 2012; Grotz et al. 1998; Hoekenga 2014). There is limited understanding of how these plant and environmental factors influence and interact to affect Zn uptake, transport, and loading into the grain. The development of a rice biofortification program therefore raises two major questions, namely the extent to which the major determinants of the concentration of grain Zn are: (1) physiological and genetic mechanisms or (2) the available soil Zn and its management. These propositions are dealt with in detail below.

6 Fertilizers as a Grain Zn, Se, and Fe Determinant

Applications of nitrogen and P could also affect Zn grain concentration of rice as N application during grain filling promotes Zn uptake and remobilization (Atique-ur-Rehman et al. 2014; Barnett et al. 2010; Bashir et al. 2006, 2012). It has been suggested that the synchronization of both Zn and N fertilization could achieve better results than the single application by avoiding the dilution effect (IFPRI, 2002). Benoist et al. (2008) stated that although high rates of P application may improve shoot growth and grain yield of rice, it may slow Zn uptake by increasing Zn adsorption to soil particles and reducing Zn absorption (Salunke et al. 2011; Sandström and Lönnerdal 1989; Sasaki et al. 2015; Seneweera and Conroy 1997). Most of the Zn use in the field is zinc sulfate fertilizer, which is the most common Zn fertilizer used in rice but has also been shown to be one of the least effective. It would be useful to investigate how other types of Zn fertilizers improve the Zn bioavailability of the plant. Further, development of improved formulations and delivery methods for Zn application in rice is urgently needed (Krishnan and Dayanandan 2003; Krishnaswami 1998; Mabesa et al. 2013; Mandal and Mandal 1986; Marschner 1995, 2012; Mäser et al. 2001). There is increasing evidence that improved growth, yield, and grain Zn concentration could be achieved through Zn

fertilization of many crops, including rice (Fageria et al. 2011, 2012; Imran et al. 2015b; Fan et al. 2001; Fernando et al. 2014). Thus, it is important to ensure that there is adequate Zn supply, either by soil Zn fertilization or foliar Zn application at critical growth stages such as heading and early grain filling (Boonchuay et al. 2013; Carl et al. 2007; Chen et al. 2000; Cunningham et al. 1994; Das and Green 2013).

On other hand, dissolved humic substances can complex Zn in soil solution, which can make Zn either less available to plants compared with sorption to cation exchange sites (common in aerobic soils) or more available to plants compared with precipitation of Zn as sulfides or carbonates (common in anaerobic soils (Seneweera and Norton 2011; Shahzad et al. 2014; Sharma et al. 2013; Shehu and Jamala 2010; Shivay et al. 2008). Higher OM also tends to drive redox potential down faster upon flooding because it provides an additional C source for microbial activity, which can cause low-redox potential precipitation reactions to happen sooner and make Zn less available to rice plants (Takahashi et al. 2012; Thilakarathne et al. 2014; Trumbo et al. 2001; Van Oosten and Besford 1996; Verma et al. 2016; Wang et al. 2011a, b; Wijesekara et al. 2009). These findings suggest that using Zn fertilizers requires a good understanding of soil conditions, but there is little information on the interaction of genotypes and fertilizer use. Recently, it has been reported that nanoparticles of titanium dioxide and ZnO boost nutrient concentration and growth of tomato plants (Behrenfeld et al. 2004; Boonchuay et al. 2013; Carl et al. 2007; Chen et al. 2000; Cunningham et al. 1994; Das and Green 2013; Imran and Khan 2015). The mechanisms and physiological impact of nanoparticle uptake and translocation should be unraveled. Irrespective of the genotypes used and any differences in Zn efficiency, removal of Zn in grain depletes soil Zn, which must be replaced (Högy and Fangmeier 2008; Högy et al. 2009; Hotz and Brown 2004; Humayan Kabir et al. 2014; Impa and Johnson-Beebout 2012).

Grain Zn and Fe concentrations in rice grains relative to other micronutrients and the negative effect on Zn may be greater if P is in higher supply (Reuter and Robinson 1997; Römheld 1991; Ruel and Bouis 1998; Sadeghzadeh 2013). These findings emphasize the importance of maintaining soil fertility to improve, or at least to maintain, existing levels of grain micronutrients, especially Zn and Fe, under e[CO_2].

7 Ways to Overcome Global Dietary Zn, Fe, and Se Deficiency

Although direct supplementation of dietary Zn may appear to be more efficient, this solution is unlikely to be adopted due to cost. Because Zn malnutrition mainly occurs where poverty is high and accessibility is difficult, the majority of those at risk of deficiency are also the least able to buy these dietary supplements (Juliano 1993; Kant et al. 2012; Katyal and Randhawa 1983; Kennedy et al. 2002; Khan et al. 2015a, b; Koch et al. 1996; Kochian 1993). A more appropriate strategy is seeking interventions that can raise the concentration of Zn in dietary staples (McDonald et al. 2002; McGrath and Lobell 2013; McNair et al. 1981; Meng et al. 2014; Myers et al. 2015).

To achieve Zn biofortified grain, greater understanding of the genetic and environmental interactions in controlling Zn homeostasis in rice is urgently needed. The global Zn nutrition goal will require the deployment of a variety of strategies including biofortification by genetic engineering or conventional breeding after screening and genetic analysis of under-utilized rice cultivars, alongside nutrition education and promotion (Wissuwa et al. 2008; Wolfgang and Bonnie 2007; Yang et al. 1998; Yoneyama et al. 2015; Yoshida and Tanaka 1969; Zee 1971). For example, increased Fe concentration of rice endosperm was achieved through overexpression of nicotianamine synthase genes (NAS) or ferritin in conjunction with NAS genes. The single-gene approaches result in a twofold increase in Fe concentration and the multi-gene approaches a sixfold increase. Further, it suggested that *OsNAS* genes, particularly *OsNAS2*, have great potential for Fe and Zn biofortification of rice (Food and Board 2001; Foster and Samman 2010; Gao et al. 2011; Graham and Rengel 1993). There is evidence that overexpression of *A. thaliana* Zn transporter in barley (*Hordeum vulgare* L.) doubled the grain Zn concentration, but there are issues with acceptance of genetically modified rice among consumers because of ecological considerations of moving barley genes into the *Oryza* gene pool. Unlike transgenic approaches for biofortification of vitamin A and Fe, it appears that conventional breeding approaches are much more practical in breeding Zn-enriched rice grain (Fageria 2013; Fageria et al. 2011, 2012; Imran et al. 2015b; Fan et al. 2001; Fernando et al. 2014; Impa et al. 2013a).

8 Conclusion

The concentration of zinc, iron and selenium in rice grains is influenced by plant-related factors (genetic factors) and environmental factors and crop management strategies (agronomical factors). Greater understanding of how these factors interact to influence grain Zn accumulation is vital for enriching Zn concentration in rice grain. Improved Zn uptake and efficient remobilization are identified as key bottlenecks for Zn biofortification. These bottlenecks should be addressed by exploiting the wide genetic diversity of rice germplasm. Micronutrient fertilization will also play an important role, especially where soils are inherently low in bioavailable nutrients. Consequently, new genetic and management strategies need to be developed to minimize this micronutrient deficiency for people whose staple diet is rice.

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Improvement of Rice Quality via Biofortification of Micronutrients

Mohammad Hasanzadeh and Nahid Hazrati

Abstract

The world's growing population and limited land resources require high intensity of food production. Human nutrition needs both macronutrients and micronutrients. The major essential micro elements for humans include manganese (Mn), iron (Fe), zinc (Zn), copper (Cu), boron (B), molybdenum (Mo), and selenium (Se). Mineral deficiency-related problems are common in the majority of the populations especially developing countries as their staple food is rice. Development of the biofortified varieties of rice by increasing the levels of biologically available nutrients and low levels of toxic elements is necessary to improve the health and nutrition of the population. Enrichment of seeds with minerals is called biofortification. This process is performed on micro nutrients such as Fe, Zn, and Se in rice via agronomic, breeding, and genetic (biotechnologic) approaches. Agronomic efforts mostly include feeding of the mother plant via traditional fertilizing or seed treatments. Selection of the biofortified genotypes via current breeding pathways is widely used. In biotechnologic approach, various genes and proteins involved in producing the seeds rich in the above mentioned elements have been identified. The present study presents an overview about various agronomic, breeding, and transgenic approaches for the biofortification of rice grains with Fe, Zn, and Se elements.

Keywords

Rice · Biofortification · Micronutrient · Quality improvement · Mineral deficiency

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1 Introduction

Cereals such as rice are important energy sources but in terms of micronutrients such as iron, zinc, and selenium, which are essential for healthy growth, exhibit rather low rates. This problem is intensified while bran—which includes some of these elements—is removed during the polishing, and lacking of these trace elements should be alleviated. The green revolution has significantly improved productivity and gained food security in developing countries, but it has not made much progress in terms of dietary diversity. Half of the global population is reported to be deficient in zinc, iron, and selenium and suffer from a variety of serious developmental and public health issues. These deficiencies are strongly associated with diets in which energy is primarily derived from the consumption of cereals, which are a poor source of minerals (Shahzad et al. 2014). To overcome the problems of micronutrient deficiency, people are recommended to be cautious about the variability of their daily diet, but most people cannot supplement their diet because of poor economic conditions.

2 Reason for Rice Biofortification

Rice is a staple food for millions of people worldwide and is of great importance for nutrient and food security. Rice is the second most widely consumed cereal after wheat in the world. Rice endosperm (starch and most edible part of rice grain) is deficient in many nutrients including vitamins, proteins, and micronutrients. The aleurone layer of rice grain is nutrient-rich but is lost during milling and polishing. Unprocessed rice becomes rigid, smelly, or unpleasant in taste (Jena et al. 2018).

New researches have been done on the importance of micronutrients, vitamins, and proteins for the purpose of biological and genetic enrichment. Procedures which farmers can grow rice for production of nutrient-rich grains in a sustainable agriculture are the only possible way to remove malnourishment of the populations in developing countries (Palanisamy 2018).

3 Zinc Importance and Deficiency

Along with other micronutrients, zinc contributes to optimum physiological functions of plants like having healthy cell membranes, improving of proteins and carbohydrates synthesis. Among all micro elements, Zn is needed in large amounts for protein production so that Zn-binding proteins reach nearly 10% of the eukaryotic proteomes. In crops such as rice, it has been found that RNA and number of ribosomes are decreased as a result of Zn deficiency leading to decline in protein synthesis (Brown et al. 1993). According to Marschner (1995), high tendency of Zn to form tetrahedral complexes with nitrogen, oxygen, and especially sulfur ligands is the reason for its metabolic functions and structural role in enzymatic reactions. Carbonic anhydrase is one of the enzymes which involve Zn as a constituent needed

for CO_2 assimilation pathway in C3 and C4 plants. In addition, ribulose 1,5-bisphosphate carboxylase (RuBPC) has been found to catalyze the initial step of CO_2 fixation during the photosynthesis in crops such as barley and rice. Also, Zn deficiency may negatively affect aldolase enzyme activity required for sucrose formation (Alloway 2008).

Zinc is deficient in 50% of the world's agricultural soils and is recognized as the world's most important micronutrient deficiency in crops. Many crops reflect Zn deficiency in various symptoms such as light green to white chlorotic and necrotic veins in mild deficiency (wheat), yellow striping of the leaves (maize), uniform chlorosis in leaves, upward dried leaves, and tip growth decrease (barley), and delayed development of the plants, chlorosis, bronzing of the leaves, and death of the seedlings at the early stages (rice).

Iron and zinc deficiency is one of the most common micronutrient deficiencies in humans, affecting 2 billion people and causing more than 0.8 million deaths annually (Dixit et al. 2019). Zinc is a key factor for several vital enzymes involved in metabolic activities (Sadeghzadeh 2013; Roohani et al. 2013). Zn deficiency causes stunting, diarrhea, poor cognitive development, loss of appetite, skin problems, and impaired immune function (Plum et al. 2010; Descalsota et al. 2018).

4 Iron Importance and Deficiency

Iron is an essential nutrient for humans and plants. Iron in animals is part of the structure of a variety of proteins (hemoglobin, myoglobin, cytochromes, flavoproteins, home-flavoproteins, transferrin, lactoferrin, ferritin, and hemosiderin). In plants, iron acts as part of many vital enzymes, such as electron transport chain cytochromes, functions in photosynthesis and electron transfer (via Fe-S clusters), in respiration, and in other important metabolic pathways (Kobayashi and Nishizawa 2012; Rout and Sahoo 2015). It also participates in the Fenton reaction, which catalyzes the generation of hydroxyl radicals (OH) and reactive oxygen species (ROS) that can cause irreversible cell damages (Wu et al. 2014; dos Santos et al. 2017). Fe is an essential component of hemoglobin and myoglobin, and its deficiency is highly associated with anemia and weakness (Abbaspour et al. 2014). Anemia caused by iron deficiency affects over 2 billion people in almost all countries, making it the most common micronutrient deficiency worldwide. The bioavailability of iron in plants is low, and in rice, this problem is exacerbated by phytate, a potent inhibitor of iron resorption, and by the absence of factors enhancing iron intake.

Among the micronutrients, iron and zinc deficiency is mostly common in developing countries (Bailey et al. 2015). Addressing micronutrient malnutrition to prevent child and women mortality is one of the UN's most important goals for sustainable development.

5 Selenium Importance and Deficiency

Selenium, in addition to being an essential ingredient for humans and animals, also has anti-cancer and antioxidant properties, which determine its use in food biofortification applications (Rios et al. 2010). Recently, interest in selenium has increased due to their presence in the glutathione peroxidase enzyme. Some studies indicate that selenium keeps the plants physiologically active for a longer period, which in some cases determines the rate of increase in crop production (Lyons et al. 2009; Ramos et al. 2011).

In a study, it has been found that rates of selenium content in rice is usually low, limiting the nutritional needs of populations depending on the amount of rice consumed in their diet (Williams et al. 2009). Selenium deficiency and low Se daily dietary intake can cause endemic diseases or other significant health problems including heart diseases, hypothyroidism, weakened immune system, and enhanced susceptibility to infections and cancer (Roman et al. 2014; Hatfield et al. 2014). For most people around the world, vegetables are an important source of plant selenium absorption. Therefore, increasing selenium concentration in food products offers an effective approach to reduce the problems of selenium deficiency in populations.

6 Different Biofortification Methods

Deficiencies of the essential elements can be removed by diet diversity, food fortification, and biofortification. Fortification is part of the post-harvest operation for increasing the content of essential micronutrients, such as vitamins and minerals (including trace elements) in food, to improve the nutritional quality of foods and to provide public health benefits with minimal risk the health, while biofortification is the process of increasing the nutritional quality of food crops that can be achieved through three main approaches, namely, agronomic, conventional breeding, and transgenic efforts, involving the use of fertilization, crop breeding, and biotechnology strategies, respectively. With recent developments in phenotyping and genetics tools, biofortification programs around the world have significantly contributed to reducing malnourishment, especially iron, zinc, and selenium in populations that are dependent on cereals as staple nutrient.

6.1 Agronomic Approaches for Biofortification

Agronomic biofortification is the deliberate use of mineral fertilizers to increase the concentration of a target mineral in crops to increase dietary intake of that mineral (Oliveira et al. 2015). The biofortification of major crops such as rice and wheat got significant scope for solving nutrient deficiency problems. Crop agronomic biofortification has advantages of rapid results, cost effectiveness, accessibility, and ease of application of agronomic approaches relative to breeding and biotechnology-based biofortification. There are different agronomic biofortification

methods including changes in nutrient utilization methods, fertilizing dosage, sensitive stages of crop nutrition, use of bio-fertilizers or plant growth promotors, and minimum or zero tillage (Jena et al. 2018).

6.1.1 Sources and Methods of Supplying Zinc and Iron

Different forms of Zn are available for plants including inorganic compounds, organic Zn, and synthetic chelates. Inorganic Zn contains ZnO, $ZnCO_3$, $ZnSO_4$, $Zn(NO_3)_2$, and $ZnCl_2$ which may be found in different soils, and due to their unique chemical features, different soil textures cause varying amounts of absorbed Zn. For instance, average rates in soils of Britain ranges from 35 mg kg^{-1} in sandy soils to 65 mg kg^{-1} in coarse loamy soils, 90 mg kg^{-1} in fine silty soils, and 106 mg kg^{-1} in clay soils (McGrath and Loveland 1992). Among which, $ZnSO_4$ is the prevalent inorganic Zn form which is easily taken up by plants. ZnO concentrated suspension can be used for foliar spraying (Moran 2004), but other forms may be more suitable. Both nitrate and sulfate anions possess high solubility by which Zn may be more mobile in plant vascular system but sulfate causes higher uptake of Zn by plant.

Iron oxide is the most abundant mineral in many soil types in forms of FeO, FeO_2, Fe_2O_3, and Fe_3O_4 which mostly are water insoluble. Among the prevalent soluble forms are $FeCl_2$, $FeCl_3$, $Fe(NO_3)_3$, and $FeSO_4$. The latter form along with iron chelates like Sequestrene™ is widely used in cultivation. $FeSO_4$ may be used as foliar spraying in circumstances which soil application of Fe is limited due to plant, soil, and environment conditions.

Different methods can be used for conventional improving of the seed Zn and Fe contents including the traditional and conventional soil fertilizing, foliar spraying, and seed treatment/coating. It has been found that most of the Fe and Zn are accumulated in the aleurone layer of the rice seed bran; therefore, the high rates of these two elements can be found in unpolished grains. The average rate of Fe in well-polished rice grain reaches 2 mg kg^{-1} seed weight (Bouis et al. 2011).

Two factors may limit Zn absorption under the anaerobic conditions which is prevalent in the rice fields. First: soil pH in paddy soils (flooding with anaerobic conditions) tends to fall. In such conditions, Fe which is in Fe^{+3} non-available (non-toxic) ferric iron is converted into Fe^{+2} (plant-available) ferrous iron due to the lack of soil oxygen, so, becomes more soluble and reaches a toxic level. Rice plant actively can inhibit taking up of the toxic Fe levels. If upland rice is cultivated, aerobic conditions is provided and Fe^{2+} is converted to the Fe^{3+} leading to Fe deficiency. Under flooding conditions, Zn which is in +2 oxidation state tends to precipitate as $ZnFe_2O_4$ and results in Zn deficiency. So, it seems that after flooding removal, Zn may be better absorbed by rice. Second: due to rather low rates of Zn application (as micronutrient against macro elements) in the soil, improving Zn content of the soil is crucial for better absorption and transportation to the grain. In case sufficient Zn is found in soil, application of this element may result in inconsistent consequences, and the majority of absorbed Zn tends to accumulate in vegetative parts rather than in grains, but in insufficient circumstances, soil Zn application may improve seed Zn content (Wissuwa et al. 2008; Johnson-Beebout et al. 2009). Despite this, genotype and soil conditions may cause plant to respond to Zn

variously (White and Broadley 2011; Jiang et al. 2008). Seed coating application with Zn seems to be more effective than soil application. Jiang et al. (2007) found that after flowering stage of rice grown in the soil with sufficient amounts of Zn along with the foliar application of this element, the majority of the accumulated element in grain originates from the soil source rather than remobilization from the leaves. Same findings in different genotypes of rice also revealed that soil Zn content is the main factor determining the seed Zn concentration (Wissuwa et al. 2008), but in soil with Zn deficiency, seed content may not be increased significantly. Opposite to this, some findings indicate that foliar spraying of Zn acts better than soil application (Wei et al. 2012; Mabesa et al. 2013). This superiority, however, depends on source of Zn or Fe, time of fertilization, and ability of genotypes to remobilize these elements from leaves to seeds (Karak et al. 2006; Cakmak 2009). When the seed coating with Zn is applied together with the foliar spraying of this element especially at milk stage, about 4-fold improvement in Zn content of the grain is achieved. It has been shown that in such a condition, phytate content of the seed which is linked to the Zn was reduced leading to a higher bioavailability of Zn to the consumer (Cakmak 2008). In another study, it has been found that seed priming with ZnSO4 increased seed yield and Zn content of rice grain (Slaton et al. 2001). Barua and Saikia (2018) illustrated that application of $ZnSO_4$ in soil along with foliar spraying significantly increased rice seed Zn content.

Other nutrient elements also can affect availability of Zn in grain. For instance, it has been revealed that if phosphorous (P) content of the soil was sufficient and absorbed by plant, it might accumulate in seeds as organic P like phytic acid (PA) - as anti-nutritional compound - and thus, might act as chelating factor leading to reduction of bioavailability of some elements like Zn (Raboy 2009; Veum et al. 2009). Inorganic phosphorous (Pi)-deficient plants may result in Zn overaccumulation in the shoots (Misson et al. 2005; Khan et al. 2014). In contrast, application of some compounds such as $CaSO_4$ by removing the soil bicarbonate content may result in pH drop so that Zn is easily available, however, in rice cultivation areas with high rates of precipitation and optimal conditions to soil oxidation; such circumstances rarely are observed (Rengel et al. 1999). Other nutrients like nitrogen (N) may negatively impact on seed content of Zn (Shi et al. 2010; Chandel et al. 2010), but this may not be true in case bio-fertilizers are consumed. In rice cultivation, Azolla and other N-fixing bacteria applications are rather prevalent, and in case applying of the Zn-solubilizing bacteria with the abovementioned microorganisms, increased grain Zn content may be resulted (Singh and Prasad 2014; Subedi and Shrestha 2015). The idea is that negative impact of N on grain Zn content relates to Zn deficiency in the soil, and decrease in Zn content is attributed to biological dilution of Zn concentration (Zhang et al. 2008); otherwise, optimum N application under adequate Zn and Fe rates promotes protein synthesis which in turn is considered as a major sink of Zn and Fe. Positive results in this regard have been observed in several crops (Kutman et al. 2010). In contrast, concomitant application of Fe and Zn does not indicate competitive effect observable in other nutrients. Reversely, they were reported to positively correlate in grain, and application of one of them did not negatively affect the absorption of the

other (Cakmak et al. 2010), and combined foliar application of these elements has resulted in improved Zn and Fe content of seed (Wei et al. 2012).

Technique of alternate wetting and drying (AWD) recommended in rice cultivation along with application of $ZnSO_4$ has been found to increase Zn content of the grain (Wang et al. 2014). Furthermore, crop rotation and intercropping of rice with cereals and legumes have been found to supply rice with Zn (Rengel et al. 1999) leading to increase in the grain Zn content. Despite little is known about re-distribution and translocation of Zn inside the plant after entering the transpiration pathway especially from vegetative organs to the grains, but based on study of stable Zn isotope, it has been found that in case Zn is applied on flag leaves during booting and anthesis, half of the absorbed Zn in rice grain takes place by remobilization from aerial parts and this element may translocate from old to new tissues (Wu et al. 2010). Same to the Zn, late foliar application of Fe has been shown to be more effective in increasing Fe content of rice grain than early season application (Phattarakul et al. 2012; Mabesa et al. 2013).

Transport of Zn and Fe in short and long distances in grasses like rice occurs both as metal complexes and ionic forms. These elements can be found both in xylem (from root to seed) and phloem sap (especially remobilization of Fe from old tissues into the seeds) which are transported mainly at the reproductive stage by using some proteins. Absorbed Fe in grain may be bound to phytate which is mainly found in aleurone layer (Persson et al. 2009) and is considered to control Zn rates in seed (Raboy 2003). Also, in excess amounts of Fe, storage of accumulated element in form of ferritin protein takes place.

6.1.2 Sources and Methods of Supplying Selenium

Selenium (Se) forms found in the soil are selenate (Na_2SeO_4), selenite (Na_2SeO_3), and organic Se, which may undergo adsorption into Fe oxides, and this ensuring availability of this element for plants (Mouta et al. 2008). Lyons et al. (2004) suggested that the use of selenium is probably the most successful example of agricultural intervention by mineral fertilizers, as selenate is highly mobile in many soil types, easily absorbed by plants, and accumulated in grains biologically in a bioavailable form such as methionine and cysteine. This element may result in grain yield increase in crops like rice (Boldrin et al. 2012) at low concentrations. In a study conducted by Wang et al. (2013), rice sprayed with 10.5 g and 21 g of selenite/hectare produced more tillers per plant, more grain per panicle, bigger grains, and higher yields. In addition, this method shortened the number of days of heading and lead to early maturity of rice. Along with yield increase, it has been found that Se as selenate form increased grain Se content of rice by soil application rather than foliar spraying (Boldrin et al. 2013). They also found that Se application by foliar spraying increased Fe content of the grain. Increasing of the Se content related to the consumption at flowering stage and earlier application might change the results. In addition to the time and method of application, Se form is important for accumulation in grain, and selenate has been found to be accumulated higher than selenite, and this has been ascribed to more easily transportation of selenate through phloem, at least in crops like potato (Poggi et al. 2000). Hu et al. (2002) found that foliar

application of Se at rates of 14–18 g kg^{-1} at heading stage increased Se content of polished mature rice grain tenfold higher than that of control. Also, Fang et al. (2009) found that foliar application of 100 g ha^{-1} of sodium selenite resulted in 55-fold increase in the Se content of the rice grain. In another study on wheat, foliar application of sodium selenate and selenite at the end of tillering at a rate of 10 g ha^{-1} significantly increased Se content of the grain (Poblaciones et al. 2014). Sun et al. (2010) showed that cultivation of rice in soil rich in Se resulted in increased rate of this element in whole grain, but reduced amounts were observed in polished seed which indicates existence of the most of the Se content in bran layer. They also revealed that Se is found in organic forms mainly Se-methionine in mature seeds. Lidon et al. (2018) reported that by applying sodium selenite as foliar spraying, Se content of rice grain was increased more relative to sodium selenate. Furthermore, high concentrations of sodium selenite and selenate increased total lipids in all genotypes and concentrations of sucrose and proteins in similar trends. Also, it has been reported that when selenium was added (0, 0.5 and 1 mg kg−1) into the soil with lead and cadmium pollution, selenium accumulation in the grains of the cultivated rice was significantly increased whereas, the lead and cadmium concentrations in different tissues were markedly decreased (Hu et al. 2014).

In contrast, de Lima Lessa et al. (2019) found that soil application of 47 and 36 g ha^{-1} of Se as sodium selenate caused increased rates of Se in the endosperm of rice grain. In addition, previous studies have also revealed beneficial effects of selenium, as it results in increased yield through increased antioxidant activity in the plant (Lyons et al. 2009).

6.2 Conventional Breeding Approaches for Biofortification

Biofortification of crops via breeding is a cost-effective and sustainable method to remove malnutrition impacts of micronutrient deficiency for people living in developing countries that cannot be supplied with Fe- and Zn-fortified foods, performed by supplementation of Zn and Fe into their staple diets. The rate of current genetic diversity for a specific trait which can be exploited by breeding varies according to the crop, and also understanding of the molecular basis of complex traits will help precisely pyramiding several genes and QTLs to develop superior and farmer-adoptive rice varieties. The genetics underlying some traits like pro-vitamin A accumulation are well known and have resulted in breeding of crops such as maize rich in pro-vitamin A (Gebremeskel et al. 2018). For many other traits, including high Fe, progress rate is slower. Iron homeostasis in plants is firmly adjusted, and biofortification usually requires exploiting these regulatory mechanisms to provide iron accumulation in given tissues (Connorton et al. 2017). While transgenic approaches focus on specific genes known to possess role in iron homeostasis, current breeding techniques lean on inheritance of the phenotypes with high iron content, along with a specific genetic marker (Rommens 2007).

6.2.1 Breeding Strategies for Developing Rice with High Nutrient Content

Since the genetic basis of the nutrients accumulation in grain is complicated with the inclusion of multiple small effect genes/QTLs and remarkably influenced by the environment, selection of the optimum breeding procedures, crossing plants, individual plant selections, and processes of the field evaluation are essential for the successful expanding of the high nutrient content rice genotypes. In the past, varieties possessing high Zn content have been exposed to cross with popular high yielding but with low Zn content rice varieties, and selection has been performed for agronomic traits in individual generations, with final fixed lines tested for yield and grain Zn content in replicated large-scale plots. This procedure is unfortunately time-consuming and causes moderate increase in Zn content, and the developed lines possess moderate yield potential. In a modified breeding program, high-Zn donor lines of rice with favorable yield potential were crossed with common high-yielding, highly-adapted but low-Zn content varieties, coupled with Zn testing in early separated lines from the F4 generation onwards for selecting the optimum agronomic traits, and it was found to accelerate process of developing of the high-Zn content variety retaining the yield potential (Swamy et al. 2016). In India and the Philippines, an improved line (IR68144-3B-2-2-3) was distinguished in a cross between a high-yielding variety (IR72) and a native tall variety (Zawa Bonday) with a high Fe content in grain of about 21 ppm in brown rice (Gregorio et al. 2000). The first rice variety enriched in Zn was developed by Harvest Plus released in 2013 by the Bangladesh Rice Research Institute (BRRIdhan 62, BRRIdhan 72, and BRRIdhan 64), which is claimed to include 20–22 ppm zinc in brown rice.

While traditional rice varieties contain higher contents of Fe than modern ones, this may be correlated with yield loss (Anandan et al. 2011). Nevertheless, introducing of the favorable traits from the wild ansestors to the developed crops via introgression of alleles has been shown to be more intended (Palmgren et al. 2015; Connorton and Balk 2019). Wild species of rice like *O. nivara, O. rufipogon, O. latifolia*, *O. granulata*, and *O. officinalis* contain high rates of Zn, about 2–3 times higher than cultivated rice, with Zn content ranging from 37 to 55 mg kg^{-1} in non-polished (brown) rice seeds (Anuradha et al. 2012a, Swamy et al. 2016). However, rice genotypes normally cultivated by farmers have relatively low rates of grain Zn content ($<$12–14 mg kg^{-1}) in polished rice and cannot meet the daily dietary requirement of this element. In this regard Garcia-Oliveira et al. (2009) identified 31 putative QTLs for Fe, Zn, Mn, Cu, Ca, Mg, P, and K contents in introgression lines resulted from crossing between elite indica cultivar Teqing and the wild-type *Oryza rufipogon*. In addition to wild relatives, the landraces, deep water rice, aromatic varieties, and colored rice are the best sources of high grain Zn.

Mutation breeding is of importance as a technique to improve Zn content in rice. Chemical and physical mutagens have been applied in mutation breeding, and mutants with high Zn content have been identified. A number of IR64 mutants produced by treating with sodium azide were reported to contain high Zn (Jeng et al. 2012; Swamy et al. 2016). It was found that three IR64 mutant lines M-IR-180,

M-IR-49, and M-IR-175 include more than 26 mg kg^{-1} Zn in polished rice compared to 16 mg kg^{-1} in IR64.

Using DNA markers for identification of QTLs has become a breakthrough in the characterization of quantitative traits. In plants, the identification of genomic regions related to quantitative traits has mostly been attained through QTL mapping (Borba et al. 2010). Using of the markers which are closely linked to QTL will allow rice breeders to perform negative selection on potentially toxic elements and positive selection on essential nutrients in grain by exploiting marker-assisted selection (MAS) (Huang et al. 2015). For most traits, homozygous and heterozygous plants cannot be distinguished by conventional phenotypic screening. MAS can be used to assist in selection of parents, increasing the effectiveness of backcross breeding, and improving sex-limited traits (Zhou et al. 2007). Also, this approach is especially useful for traits strongly affected by environment and genotype interactions (Descalsota-Empleoa et al. 2019).

Forthermore genome-wide association studies (GWAS) and Biparental mapping have been performed to map QTLs for different traits in rice and for determining chromosomal regions and specific alleles, linked to Fe and Zn content in crops (Norton et al. 2014; Zhang et al. 2014, 2017)

Biparental populations, such as recombinant inbred lines (RILs), doubled haploids (DHs), backcross inbred lines (BILs), and introgression lines (ILs), have been found to be effective for detecting major-effect QTL (Zhang et al. 2014; Xu et al. 2015; Yu et al. 2015; Kumar et al. 2018). Major QTL precisely can be transferred to different genetic backgrounds through marker-assisted breeding approaches, causing faster development of rice varieties (Collard and Mackill 2008).

Several reports have revealed the usefulness of DH populations for identifying QTL for minerals content of grain. In this sense, six QTL for Mn, Fe, and Zn were identified in different DH populations of rice (Stangoulis et al. 2007). Also, 23 QTLs were identified in two environments for K, Ca, Mg, Fe, P, Mn, and Zn (Du et al. 2013). Swamy et al. (2018) identified 59 QTLs for a number of biofortification traits such as Ca, Cu, Mg, Mn, Mo, Fe, and Zn. Using RILs, 14 QTL were detected for grain Fe and Zn contents, and the candidate genes which were close to QTLs, including *OsYSL1* and *OsMTP1* for Fe; *OsARD2*, *OsIRT1*, *OsNAS1*, and *OsNAS2* for Zn; and *OsNAS3*, *OsNRAMP1*, and *APRT* for Fe and Zn, were identified (Anuradha et al. 2012b). Zhang et al. (2014) also found 134 QTLs associated with the concentration of individual elements using RILs in unmilled rice grains.

Hu et al. (2016) applied inbred lines (BILs) derived from an interspecific cross of *Oryza sativa* × *O. rufipogon* to detect quantitative trait loci (QTLs) for mineral nutrient contents. A total of 24 QTLs for mineral element contents were identified, including 17 for brown rice only, 5 for milled rice only, and 2 QTLs for both brown and milled rice, and all the 7 QTLs detected for the mineral contents in milled rice and 13 out of the 19 QTLs for the contents in brown rice had the enhanced alleles derived from wild parent *O. rufipogon*. The authors detected six QTLs for Zn content, including one (qZn10) for both the brown and milled rice, four (qZn4, qZn6, qZn3, and qZn12) for brown rice only, and one (qZn7) for milled rice only. In addition, three QTLs were detected for Fe content including qFe3, qFe6, and qFe9

which all of them were responsible for the Fe content in brown rice. They also reported one detected QTL, qSe7 for Se content that showed significant effects on Se contents in brown and milled rice with the enhanced allele derived from wild parent. Moreover, a total of five backcross inbred lines (BILs) in rice with moderate Fe and high Zn content in grains coupled with increased yield were identified by Dixit et al. (2019), and each of the five BILs with high grain Fe and Zn content involves at least one QTL related to grain Zn/Fe.

GWAS (genome-wide association study) possess several advantages over biparental mapping such as high mapping resolution and identification of the multiple and rare alleles (Nordborg and Tavaré 2002). GWAS is able to detect QTLs playing a role in increasing grain Fe, Zn levels, and several other mineral elements (Norton et al. 2010; Zhang et al. 2014) and could identify the effect of many different single-nucleotide polymorphisms (SNPs) in unrelated populations (Mitchell-Olds 2010). Content of the five nutrient elements was investigated in 378 brown rice accessions, and association mapping was applied to identify QTLs responsible for the element variations, and in this regard, 20 QTLs including some previously reported and new candidate loci were identified (Huang et al. 2015). During an experiment, 72 loci were distinguished by genome-wide association analysis for 17 mineral elements in a panel of 529 rice accessions (Yang et al. 2018).

On account of rather scarcity of genetic markers in the genome, QTL often corresponds to large chromosomal regions, sometimes over a hundred genes. Refining the region and identifying the particular involved genes can challenge the next step, and a combination of GWAS and QTL mapping aids this process. Furthermore, as the impact of a particular QTL can be modulated by environmental factors and vary between studies (Garcia-Oliveira et al. 2018), so meta-QTL study for grain Fe and Zn traits of already reported QTLs can provide better understanding of the QTL effect in different genetic backgrounds and for locate major effect QTLs. This analysis can be considered as part of validation of QTLs by understanding the confidence interval (CI) for the already reported QTLs (Goffinet and Gerber 2000). Combining different studies relating to QTLs for grain Fe and Zn content to identify meta-QTL is a very promising approach to realize the QTL effect in different genetic backgrounds and to locate QTL position on the consensus map (Dixit et al. 2019).

In recent years, multi-parent advanced generation inter-cross (MAGIC) populations have become common genetic resources for mapping and developing breeding lines with multiple favorable traits (Meng et al. 2016).

MAGIC populations possess a rather wide genetic background without significant population structure, which is a major constraint in association mapping using diversity panels. The further-refined MAGIC Plus population with additional generations of intermating has increased levels of recombination and thus greater mapping resolution (Bandillo et al. 2013), so MAGIC Plus rice lines can be powerful genetic resources to facilitate QTL analyses/gene discovery with high mapping resolution for both complex and simple traits (Descalsota et al. 2018). At IRRI, several MAGIC populations such as MAGIC-japonica, MAGIC-indica, and MAGIC-global (utilizing crosses between indica and japonica MAGIC lines) have

been developed (Bandillo et al. 2013), and these are good resources for selecting high Zn lines and also provide opportunities to select transgressive segregants for high Zn content. Through GWAS of a 144-strong multi-parent advanced generation inter-cross (MAGIC) Plus population, loci associated with grain Fe content have been identified in rice, including Fe homeostasis genes such as nicotianamine (NA) synthase OsNAS3, vacuolar Fe transport *OsVIT1*, and also *OsMTP6*, *OsMT2D*, and *OsNRAMP7* which were co-located with QTLs for Fe and Zn (Descalsota et al. 2018).

Dixit et al. (2019), during a study on identification of genomic region (s) responsible for high iron and zinc content in rice, detected a total of two major QTLs for Fe and three QTLs for Zn, based on the multiseason phenotypic data, together with genotypic ones. Moreover, four consistent QTLs for Fe and two QTLs for Zn content were identified via comparative analysis across the two seasons and also identified potential candidate gene(s) such as *OsPOT*, *OsZIP4*, *OsFDR3*, and *OsIAA5* which were described previously to influence grain Fe and Zn content.

These results have revealed that there are multiple loci distributed throughout the genome and that they induce minor to moderate effects on the accumulation of different mineral elements in rice grain. Even though there are a few major loci, they have not been used widely in marker-assisted selection (MAS) for developing the high-yielding nutritious rice. On the other hand, there are several studies which report a positive correlation between Fe and Zn (Paltridge et al. 2012; Rao et al. 2014), but also strong negative correlation of grain Fe and Zn content with yield in rice was detected (Norton et al. 2010; Dixit et al. 2019). Therefore, identifying high Fe/Zn donor lines with acceptable yield potential is necessary by designing favorable breeding approaches, selection schemes, and evaluation processes for the successful development and release of high-yielding varieties with high Fe/Zn contents (Suwarto 2011; Dixit et al. 2019).

In staple grains such as rice, it is complicated to improve some complex traits such as iron content and a substance like vitamin A using conventional breeding strategies, as there are no natural rice varieties rich in these nutrients. In other words, there is enough genetic variability for grain Zn in the polished rice within cultivated rice germplasm, but not for grain Fe (Swamy et al. 2016). Hence, it is possible to breed for high-Zn rice via breeding approaches by exploiting high-Zn donors, while a transgenic approach is probable for developing the high-Fe rice (Trijatmiko et al. 2016). Furthermore, though several high-Zn content rice varieties have been successfully developed and released for cultivation by conventional breeding approaches, this process is slow on account of the lack of field-based phenotyping techniques, complex genetic nature of high grain Zn, firmly linked markers, and significant environmental and genotype interactions (Zhang et al. 2014; Descalsota et al. 2018). Therefore, agricultural biotechnology methods, and genetic engineering (GM), represent a very valuable and complementary strategy for the developing more nutritious crops.

6.3 Biotechnological Approaches for Biofortification

Biofortification by breeding approach has been achieved in crops and specified components when genetic variation is available in the gene pool of the targeted crop for the desired trait. When genetic variation is not available, genetic transformation is the better option. The transgenic-based approach has the benefits that a useful gene after its discovery can be used to target several products (Garg et al. 2018). Harvest Plus have also recently pointed that conventional seed breeding techniques are not always successful with increasing iron and zinc. They have noted that, using transgenesis to produce gmo-crops, they got success in these traits in rice only through precision farming (Ebron 2016).

Not only increasing the iron and zinc (Trijatmiko et al. 2016) but also folate (Storozhenko et al. 2007; Blancquaert et al. 2015) in poor crop products (Paul et al. 2017) have all extremely importance for human healthy, and they have all been achieved applying gmo technology, where conventional breeding was not efficient (Dubock 2017). On the other hand, genetic engineering technologies are able to provide a more efficient and reliable way of examining the relationship between genotype and phenotype compared to agronomic and conventional breeding strategies (Gaj et al. 2013; Yin et al. 2017; De-Xian Kok et al. 2018).

6.3.1 Biofortification via Genetic Engineering for Iron and Zinc

Genes have been investigated by researchers at all stages of iron homeostasis (uptake, transportation, storage, and regulation) (Masuda et al. 2013b; Vasconcelos et al. 2017; Kawakami and Bhullar 2018). Among the available methods for rice biofortification for Fe, transgenic methods can increase iron levels in rice grains more efficiently. Below, seven transgenic approaches usually used to increase the Fe content of rice seeds through the Fe biofortification of rice as well as combination of different transgenic approaches, which are successfully applied to enhance the iron and zinc concentrations in rice grain, have been described. Also, the introduced genes and applied promoters, rice cultivars, and increased amount of nutrient(s) have been presented in Table 1.

6.3.1.1 Increasing Seed Iron Content by Introducing Fe Storage Protein, *Ferritin* Gene, *SoyferH1*, *SoyferH2*, or *Pvferritin*

In plants, iron is stored as ferritin form in plastids and mitochondria (Duy et al. 2011; Vigani et al. 2013), or even in the cell vacuole (Gollhofer et al. 2014), which is a large non-toxic storage form of iron that can release Fe for metabolic functions when needed. Being ubiquitous, iron storage protein ferritin reserves about 4500 Fe atoms in bioavailable form (Darbani et al. 2013). The iron complex in soybean ferritin is readily accessible to the human body through the mechanism of iron uptake in the intestine (Theil 2011). Therefore, the first approach in iron biofortification is to enhance ferritin expression by introducing soybean *ferritin* genes (*SoyferH1* and *SoyferH2*) into rice.

However, introduction of the *SoyferH2* in rice plants is preferred because *SoyferH1* is more sensitive to protease digestion than *SoyferH2*, causing alterations

Table 1 Iron biofortification strategies in rice, introduced genes and applied promoters, rice cultivars, and increased amount of iron

Strategy	Gene promoter	Rice cultivar	Fold of increase	References
Improving iron storage via *ferritin* genes	*OsGluB1* pro-*SoyferH1*	*Japonica* cv. Kitaake	2-fold Fe (polished grain)	Goto et al. (1999)
	OsGluB1 pro-*SoyferH1*	*Japonica* cv. Taipei 309	2.2-fold Fe (brown grain)	Lucca et al. (2002)
	OsGluB1 pro-*SoyferH1*	*Indica* cv. IR68144	3.7-fold Fe (polished grain)	Vasconcelos et al. (2003)
	OsGluB1 pro-*SoyferH1*	*Japonica* cv. Kitaake	3-fold Fe (brown grain)	Qu et al. (2005)
	OsGluA2 pro-*Osfer2*	*Indica* cv. Pusa-Sugandh II	2.1-fold Fe 1.37-fold Zn (polished grain)	Paul et al. (2012)
	OsGluB4 pro-*SoyferH1*	*Indica* cv. IR64	3.4-fold Fe (polished grain)	Oliva et al. (2014)
Enhancing iron transportation via *NAS* gene	Maize *Ubiquitin* pro-*OsNAS3*	*Japonica* cv. Dongjin	2.9-fold Fe 2.2-fold Zn (polished grain)	Lee et al. (2009b)
	pGluB-1 pro-*HvNAS1*	*Japonica* cv. Tsukinohikari	1.5–2-fold Fe 1.5–2-fold Zn	Usuda et al. (2009)
	35S pro-*HvNAS1*	*Japonica* cv. Tsukinohikari	2.3-fold Fe 1.5-fold Zn (polished grain)	Masuda et al. (2009)
	35S pro-*OsNAS2*	*Japonica* cv. Nipponbare	4-fold Fe 2-fold Zn (polished grain)	Johnson et al. (2011)
	Maize *Ubiquitin* pro-*OsNAS2*	*Japonica* cv. Kitaake	3-fold Fe 2.7-fold Zn (polished grain)	Lee et al. (2012)

(continued)

Table 1 (continued)

Strategy	Gene promoter	Rice cultivar	Fold of increase	References
	Maize *Ubiquitin* pro-*OsNAS1*	*Oryza sativa* L. cv EYI 105	2.0-fold Fe 2.5-fold Zn (polished grain)	Díaz-Benito et al. (2018)
Enhancing iron influx via *OsYSL2* gene	*OsSUT1* pro-*OsYSL2*	*Japonica* cv. Tsukinohikari	4.4-fold Fe (polished grain)	Ishimaru et al. (2010)
Enhancing iron uptake and translocation via *IDS3* gene	*35S* pro-barley 20-kb *IDS3* genome fragment	*Japonica* cv. Tsukinohikari	1.4-fold Fe 1.35-fold Zn (polished grain)	Masuda et al. (2008)
	35S pro-barley 20-kb *IDS3* genome fragment	*Japonica* cv. Tsukinohikari	1.3-fold Fe (brown grain)	Suzuki et al. (2008)
Enhancing Fe uptake and transportation via *OsIRT1* gene	*Ubiquitin* pro-*OsIRT1*	*Japonica* cv. Dongjin	1.1-fold Fe (brown seeds)	Lee and An (2009)
Enhancing Fe uptake and transportation via *OsYSL15* gene	*OsActin1* pro-*OsYSL15*	*Japonica* cv. Dongjin	1.3-fold Fe (brown seeds)	Lee et al. (2009a)
Enhancing Fe uptake and translocation via *OsIRO2* gene	*35S* pro-*OsIRO2*	*Japonica* cv. Tsukinohikari	3-fold Fe 1.4-fold Zn (brown seeds)	Ogo et al. (2011)
Enhancing iron translocation via knock-down of *OsVITs* genes	*OsVIT1* or *OsVIT2* T-DNA insertion line	*Japonica* cv. Zhonghua11 and *Japonica* cv. Dongjin	1.4-fold Fe (brown grain)	Zhang et al. (2012)
	OsVIT2 T-DNA insertion line	*Japonica* cv. Dongjin	1.8-fold Fe (polished grain)	Bashir et al. (2013)

in its structure (Masuda et al. 2013b; Trijatmiko et al. 2016). Interestingly, rice plants introduced with single soybean *ferritin* gene did not show an increase in iron content of grain (Qu et al. 2005; Masuda et al. 2013b). This suggests that the expression of *ferritin* genes depends on soil composition and that overexpression of *ferritin* genes as a single transgene approach may be insufficient to cope with iron

deficiency (Qu et al. 2005; Lephuthing et al. 2017). Not only the soybean storage gene was used to improve the grain iron levels, but the effect of overexpression of rice *ferritin* gene (*OsFer2*) was also analyzed (Paul et al. 2012). *A. thaliana* has four ferritin-encoding gene homologs (*AtFER1* to *AtFER4*), whereas two of these are found in *O. sativa* (*OsFER1* and *OsFER2*) (Silveira et al. 2009). Enhancement of Fe accumulation in rice by *ferritin* gene expression under the control of endosperm-specific promoters is an important strategy in Fe biofortification. Studies on this strategy demonstrated that overexpression of *ferritin* in several products increased Fe concentration as well as its bioavailability (Qu et al. 2005; Aluru et al. 2011; Borg et al. 2012).

6.3.1.2 Increasing Iron Transportation Within the Plant by *NAS* Overexpression

The second approach involves increasing iron transfer to the plant through overexpression of genes contributed to nicotianamine (NA) biosynthesis such as *nicotianamine synthase* (*NAS*). NA has been found to be biosynthesized from methionine by NAS and S-adenosyl methionine synthase (SAMS) and this substance has been introduced as a chelating agent for some cations like Zn (II) and Fe (II) (Higuchi et al. 1994). It has been revealed that NA is synthesized and utilized by all higher plants for transporting of the iron and other cations internally (Takahashi et al. 2003; Hell and Stephan 2003).

Rice has three *NAS* genes from which the expression of *OsNAS1* and *OsNAS2* but not of *OsNAS3* is strongly stimulated in response to Fe and Zn deficiencies. Recently, it has been found that the expression of *OsNAS3* is strongly induced by excess Fe in most rice tissues, particularly older leaves, suggesting that it may play a vital role under extreme Fe conditions (Aung et al. 2019). The results indicate that NAS and NA play an important role in long-distance transportation of iron in rice, in addition to their roles in phytosiderophore synthesis (Masuda et al. 2013a). Numerous studies have reported disruption of internal metal transportation in NA-defective plants. Nicotianamine is not only concerned in long distance Fe transportation in rice but also serves as a substrate for producing of deoxymugineic acid (DMA) by NA aminotransferase (NAAT) and DMA synthase (DMAS). Therefore, *HvNAS1* overexpressing not only increased the NA content but also increased the deoxymugineic acid (DMA) content in rice (Masuda et al. 2009). Overexpression of *OsNAS1* and *HvNAATb* in rice has been shown to lead to a 29-fold increase in DMA and a 4-fold increase in iron concentration in polished rice grain (Banakar et al. 2017).

6.3.1.3 Increasing Fe Flux to Seed by Expressing Fe(II)-NA Transporter Gene *OsYSL2*

Another approach to increase iron transportation from the phloem into the developing seeds involve overexpress of the *YSL2* transporter in rice which acts in uptake process of Fe(II)–NA from the rhizosphere. A total of 18 different *YSL* (yellow stripe-like) genes (*OsYSL1-YSL18*) were identified by Koike et al. (2004) in rice. Rice metal-nicotianamine transporter, *OsYSL2*, is required for the long-distance

transportation of iron and accumulation of iron in endosperm (Koike et al. 2004; Ishimaru et al. 2010). The result of various studies strongly suggests that *OsYSL2* plays an important role in loading of iron into the rice grains. In this regard, Ishimaru (2010) illustrated that inhibition of the *OsYSL2* gene in rice caused reduction in grain iron content by 18% (brown) and 39% (polished), respectively, by increasing iron accumulation in roots compared to the wild types. Ishimaru et al. (2010) also reported that it is important to use the rice sucrose transporter promoter (*OsSUT1*) for enhancing iron accumulation in polished rice, since no effect was observed with *35S* promoter.

6.3.1.4 Increase in Iron Uptake and Transfer by Overexpressing *IDS3* Gene

The fourth approach to iron biofortification involves enhancing iron uptake and translocation through introduction of MAs biosynthesis responsible genes. Roots of cereals have been reported to exudate mugineic acid family phytosiderophores (Mas) as natural chelating agents for Fe (III) which cause Fe uptake from the rhizosphere (Mihashi and Mori 1989). Among cereal plants, barley is highly tolerant to iron deficiency and involves a series of MAs biosynthetic genes, including *HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, *HvDMAS1*, *IDS2*, and *IDS3*, the expression levels of which are upregulated in barley roots under iron deficiency conditions (Nakanishi et al. 2000; Bashir et al. 2006). In contrast, rice lacks the ability to synthesize other types of MAs except DMA because rice does not contain *IDS2* and *IDS3* genes (Masuda et al. 2013a).

Three transgenic rice lines, which were transformed with the barley genome fragments involving mugineic acid synthase genes of *HvNAS1* gene or *HvNAS1* and *HvNAAT-A*, *HvNAAT-B* genes or *IDS3* gene, were produced (Masuda et al. 2008; Suzuki et al. 2008). Fe concentrations in the seeds of transgenic lines of rice were analyzed after cultivation in Fe-sufficient or low Fe-available soils (Masuda et al. 2008; Suzuki et al. 2008). The *IDS3* rice lines had a 1.3-fold higher concentration of iron in brown grains than in non-transgenic rice, a 1.4-fold greater in polished grains after cultivation in iron-rich soil (Masuda et al. 2008), as well as 1.3-fold greater in brown grains in low Fe conditions (Suzuki et al. 2008).

Higuchi et al. (2001) produced transgenic rice lines containing the barley *HvNAS1* gene and reported *HvNAS1* expression in both Fe-deficient roots and leaves and also in roots with sufficient Fe. Takahashi et al. (2001) introduced barley *HvNAAT-A* and *HvNAAT-B* genome fragments into rice and noted the increased Fe deficiency tolerance and well growth of transformants in alkaline soil. Similar results were obtained by Kobayashi et al. (2001) by introducing the barley *IDS3* gene into rice that led to secretion of a hydroxylated form of DMA, mugineic acid.

6.3.1.5 Increasing Iron Uptake from Soil by Fe Transporter Gene *OsIRT1* or *OsYSL15*

Another approach to enhance iron uptake is the overexpression of the *OsIRT1 or OsYSL15 iron transporter genes.* Phytosiderophores (PS), which are results of the function of nicotianamine synthase (NAS), nicotianamine aminotransferase (NAAT), and deoxymugineic acid synthase (DMAS), can bind to Fe^{3+} forming the

soluble complex Fe(III)-PS, and these complexes in the rhizosphere can be taken up into root cells through the action of *YSLs* (Nozoye et al. 2011). Wairich et al. (2019) noted that both *OsYSL15* and *OrYSL15* genes were upregulated in roots of Fe-deficient plants. Since YSL15 transport is essential for the uptake of Fe (III) phytosiderophore into root cells, it is clear that this strategy is induced in both *O. sativa* and wild *O. rufipogon*. Furthermore, Lee et al. (2009a) found that using *OsActin1* promoter, the concentration of iron in brown seeds increased by approximately 1.3-fold by overexpressing of *OsYSL15* compared to non-transgenic rice.

It was shown that *O. sativa* may induce the *OsIRT1* and *OsIRT2*, Fe^{2+} transporters under Fe deficiency (Ishimaru et al. 2006; Walker and Connolly 2008). Based on these evidences, it has been suggested that *OsIRT1* is involved in Fe uptake in rice, allowing rice roots to acquire Fe^{2+} from the soil. This would be an adaptation to paddy fields where Fe^{2+} is much more abundant than Fe^{3+} and where rice is frequently cultivated (Ishimaru et al. 2006). Lee and An (2009) developed transgenic rice expressing the rice ferric ion transporter gene *OsIRT1* under the control of the *ubiquitin* promoter. This rice showed a 13% increase in iron concentration in the brown seeds, while the concentration in the leaves increased 1.7-fold. Wairich et al. (2019) reported that the combined strategy to Fe uptake observed in rice, based on *OsYL15* as Fe^{3+} phytosiderophore transporters and *OsIRT1* as Fe^{2+} transporters, is not an evolutionary novelty limited to *O. sativa* but also is present in species of the *Oryza* genus.

In addition, iron-regulated transporter *OsIRT1* overexpression results in Fe, Zn, and Cd uptake and accumulation in rice tissues (Lee and An 2009). Therefore, *OsIRT1* is characterized by Fe as well as Zn uptake in rice (Lee and An 2009; Nouet et al. 2011). After transported into cytosol, Zn can be accumulated into the vacuoles via transporter *OsZIP1* (Yoneyama et al. 2015). *OsZIP1* located intonoplast mediates influx of Zn into vacuoles. MAs are also characterized for their role in Zn uptake in plants (Bashir et al. 2012).

6.3.1.6 Increased Iron Uptake and Iron Transfer by Overexpressing the *OsIRO2* Gene

Previously, the Fe deficiency-inducible basic helix-loop-helix transcription factor gene *OsIRO2*, which binds specifically to CACGTGG sequence, has been characterized (Ogo et al. 2006). *OsIRO2* is responsible for the regulation of genes involved in Fe homeostasis in rice as the induction of various Fe deficiency-induced genes in rice roots, including genes involved in mugineic acid (MA) biosynthesis (*OsNAS1*, *OsNAS2*, *OsNAAT1*, and *OsDMAS1*) and Fe (III)–MA transport (*OsYSL15*), all are controlled by *OsIRO2* expression. *OsIRO2* also induced the expression of Fe deficiency-inducible transcription factor genes harboring *OsIRO2*-binding core sequences in their promoter regions (Ogo et al. 2007). *OsIRO2* itself contains multiple *IDEF1*-binding core sequences in its promoter region and is regulated by *IDEF1* (Kobayashi et al. 2007). *IDEF1* expression is correlated with that of *OsIRO2*, *OsIRO3, OsYSL15*, *OsIRT1*, *OsYSL2*, *OsNAS1*, *OsNAS2*, *OsNAS3*, and *OsDMAS1* just after the beginning of the Fe deficiency,

proposing that *IDEF1* is essential for the early beginning of Fe deficiency response (Kobayashi et al. 2009).

Ogo et al. (2011) noted that *OsIRO2* overexpressing rice showed improved tolerance to low Fe in calcareous soil and increased iron content in shoots and also accumulated greater amount of iron in grain than control rice. Masuda et al. (2017) recently produced a Fe deficiency-tolerant rice lines possessing *OsIRT1* promoter-*refre1/372* and *35S* promoter-*OsIRO2*. They reported that the introduction of the combination of two genes (*refre1/372* and *OsIRO2*) was more effective to provide Fe deficiency tolerance in rice than the single introduction of each gene in early and middle-late growth stages under calcareous soil conditions. The produced lines also displayed higher yield (ninefold) than non-transformed line.

6.3.1.7 Increase in Fe Translocation by Silencing of the *OsVIT1* or *OsVIT2*

In rice, it has been found that the *OsVIT1* and *OsVIT2* genes encode the vacuolar membrane transporters involved in storage of Zn^{2+} and Fe^{2+} in vacuoles of the flag leaves (Kim et al. 2006). High level expression of the *OsVIT* genes were detected in flag leaves whereas, these genes were found to be expressed ubiquitously in different parts of the plants at low levels. Also, it was found that disruption of the *VIT* orthologues increased Fe and Zn accumulation in brown rice seeds by 1.4 fold whereas, decreased Fe and Zn accumulation by 0.8 fold in flag leaves (Zhang et al. 2012). Also, Bashir et al. (2013) found that knockdown of *OsVIT2* increased Fe and Zn accumulation in brown and polished rice grains by 1.3-fold and 1.8-fold, respectively.

Zhang et al. (2012) and Bashir et al. (2013) proposed this as a novel strategy for producing iron-biofortified rice so that disruption of *OsVIT1* or *OsVIT2* increased iron translocation between the source and sink organs. They also noted that this approach should be avoided in cadmium-contaminated soils because cadmium content was also increased following *VIT* knockdown in rice.

6.3.2 Multiple Transgene Approach Used for Iron and Zinc Biofortification in Rice

Multiple gene manipulation has been successfully performed in rice. Although there have been significant advances in revealing the mechanisms related to Zn and Fe homeostasis in model plants, detailed knowledge is still lacking. It is noteworthy to emphasize that different genes controlling Fe and Zn homeostasis in cereal grains have been characterized, but their impact on genotypic variation for accumulation of the mentioned minerals in the grain remains unknown. Remarkable correlations between grain Fe and Zn contents have been reported in wheat (Velu et al. 2011), rice (Stangoulis et al. 2007), and sorghum (Kumar et al. 2009). This proposed that these traits might possess some common genetic mechanisms causing their accumulation in grains. For example, some common members of the *ZIP* family, which are contributed to the transportation of Fe and Zn along with other varieties of divalent cations, have been reported. *OsZIP1*, *OsZIP2*, *OsZIP3*, and *OsZIP4* are associated with zinc homeostasis (Ishimaru et al. 2005, 2007), and *OsZIP7a* and *OsZIP8* might encode Fe and Zn transporters, respectively (Yang et al. 2007). Furthermore, there is

arising evidences for role of NA in Zn homeostasis (Takahashi et al. 2003; Haydon and Cobbett 2007). It has been proposed that in addition to iron translocation, DMA phytosiderophore plays a role in long distance transportation of Zn (Suzuki et al. 2008; Wirth et al. 2009).

Biofortified rice was generated with increased Fe concentration by Masuda et al. (2012) using multiple transgene approach. The introduced genes, *ferritin*, *HvNAS1*, and *OsYSL2*, functioned synergistically and increased the concentration of NA and DMA in the plant by overexpression of the *NAS* gene and correspondingly accelerated the formation of Fe(II)–NA or Fe(III)–DMA and transportation of Fe (II)–NA or Fe(III)–DMA in the phloem. Additionally raised the accumulation of Fe storage protein ferritin as well as improved iron translocation by overexpressing the iron(II)–nicotianamine transporter *OsYSL2* in endosperm of rice. As a result, they noted that the transgenic lines produced higher levels of iron by 6-fold in the greenhouse and higher levels of zinc by 1.6-fold, while yield remained similar to conventional rice. The authors also concluded that the introduction of multiple genes involved in iron and zinc homeostasis is more effective than the introduction of a single gene for iron biofortification. Afterward, Masuda et al. (2013b) successfully increased iron and zinc accumulation even more by enhancing the uptake and transportation of iron in *ferritin* and *mugineic acid* expressed rice plants. In this case, the authors generated transgenic plants expressing the barley nicotianamine synthase gene (*HvNAS1*), two nicotianamine aminotransferase genes (*HvNAAT*-A and *HvNAAT*-B), and a mugineic acid synthase gene (*IDS3*) to increase mugineic acid production in rice plants along with the soybean ferritin gene (*SoyferH2*) driven by two endosperm-specific promoters. Transgenic lines expressing both *ferritin* and *mugineic acid* biosynthetic genes showed signs of Fe deficiency tolerance in calcareous soils. In this sense, it has been shown that transgenic plants increased iron concentrations by 2.5-fold in Fe-deficient soil. In addition, transgenic lines grown under Fe-sufficient conditions increased iron accumulation by fourfold as much as lines grown in both commercially supplied soil (Fe-sufficient conditions) and calcareous soils (Fe-deficient conditions).

Similarly, Aung et al. (2013) generated a transgenic line of rice overexpressed the nicotianamine synthase (*HvNAS1*) gene, the Fe(II)-nicotianamine transporter (*OsYSL2*) gene, and also the Fe storage protein (*SoyferH2*) gene (Table 2). Generated transgenic rice plants accumulated more than 3.4-fold higher Fe concentrations, as well as 1.3-fold higher zinc concentrations compared to non-transformed rice, thus illustrating that transgenic rice biofortified for increased iron content could address both iron and zinc micronutrients deficiency. Trijatmiko et al. (2016) also used a transgenic approach to show that plants which express soybean ferritin (*SferH-1*) and rice nicotianamine synthase (*OsNAS2*) genes demonstrated enrichment of endosperm in Fe and Zn. Likewise, the authors applying Caco-2 cell assay illustrated that increased Fe and Zn are bioavailable. Singh et al. (2017) developed rice lines expressing *Arabidopsis Nicotianamine Synthase 1* (*AtNAS1*), bacterial *Carotene Desaturase* (*CRTI*), bean *Ferritin* (*PvFerritin*), and maize *Phytoene Synthase* (*ZmPSY*) in a single genetic locus in order to increase Fe, Zn, and β-carotene content in endosperm of the rice. NAS catalyzes the synthesis of

Table 2 Multiple transgene approaches were used for iron and zinc biofortification in rice

Gene promoter used	Rice cultivar	Fold of Fe and Zn increase	References
OsGlb1 pro-*Pvferritin* *35S* pro-*AtNAS1* *OsGlb* pro-*Afphytase*	*Japonica* cv. Taipei 309	6.3-fold Fe 1.5-fold Zn (polished grain)	Wirth et al. (2009)
OsGluB1 pro-*SoyferH2* *OsGlb1* pro-*SoyferH2* *OsAct* pro-*HvNAS1* *OsSUT1* pro-*OsYSL2* *OsGlb1* pro-*OsYSL2*	*Japonica* cv. Tsukinohikari	6-fold Fe 1.6-fold Zn (brown grain)	Masuda et al. (2012)
OsGluB1 pro-*SoyferH2* *OsGlb1* pro-*SoyferH2* *HvNAS1, HvNAAT-A, -B* and *IDS3* genome fragments	*Japonica* cv. Tsukinohikari	4-fold Fe 0.3-fold Zn (polished grain)	Masuda et al. (2013b)
OsGluB1 pro-*SoyferH2* *OsGlb1* pro-*SoyferH2* *OsAct* pro-*HvNAS1* *OsSUT1* pro-*OsYSL2* *OsGlb1* pro-*OsYSL2*	Tropical *Japonica* cv. Paw San Yin	3.4-fold Fe 1.3-fold Zn (polished grain)	Aung et al. (2013)
MsENOD12B pro-*AtIRT1* *OsGlb1* pro-*Pvferritin* *35S* pro-*AtNAS1* *OsGlb* pro-*Afphytase*	*Japonica* cv. NFP	2.2-fold Fe 1.5-fold Zn (polished grain)	Boonyaves et al. (2016)
GluA2 pro-*SoyferH1* *35S* pro-*OsNAS2*	*Indica* cv. IR64	7.5-fold Fe 3.3-fold Zn (polished grain)	Trijatmiko et al. (2016)
Native *AtIRT1* pro-*AtIRT1* *OsGlb1* pro-*Pvferritin* *35S* pro-*AtNAS1*	*Japonica* cv. Nipponbare	4.7-fold Fe 1.8-fold Zn (polished grain)	Boonyaves et al. (2017)
CaMV 35S pro-*AtNAS1* *OsGlobulin* pro-*Pvferritin* *OsGlutelin1* pro-*paCRTI* *OsGlutelin* pro-*ZmPSY*	*Japonica* cv. Nipponbare	3.3-fold Fe 1.28-fold Zn (polished grain)	Singh et al. (2017)
OsIRT1 pro-*refre1/372* *35S* pro-*OsIRO2*	*Japonica* cv. Tsukinohikari and Tachisugata	12-fold Fe (brown grain)	Masuda et al. (2017)
ZmUbiquitin-1 pro-OsNAS1 *ZmUbiquitin-1 pro-HvNAATb*	*Oryza sativa* L. cv EYI 105	4-fold Fe 4-fold Zn (polished grain)	Banakar et al. (2017)
ZmUbiquitin pro-*OsNAS1* *ZmUbiquitin* pro-*HvNAATb*	*Oryza sativa* L. cv EYI 105	2.9-fold Fe (polished grain)	Díaz-Benito et al. (2018)

nicotianamine (NA), which acts as a precursor of deoxymugeneic acid (DMA) for Fe and Zn chelating either for short- or long-distance transportation. Ferritin provides efficient storage for iron ions. PSY catalyzes the conversion of GGDP to phytoene, and CRTI improves function of the desaturases required for the synthesis of β-carotene from phytoene. As a result, all transgenic rice lines acquire significantly

increased β-carotene, iron, and zinc content in the polished rice grains. Banakar et al. (2017) generated transgenic rice plants that accumulated iron and zinc in endosperm expressing nicotianamine (NA) and 20-deoxymugenic acid (DMA). Transgenic rice plants were capable of accumulating up to twofold more zinc (Zn) and fourfold more iron (Fe) in rice endosperm, along with lower levels of cadmium compared to wild-type plants that result in a reduced toxicity and also high nutritional value of produced rice seed (Table 2).

Díaz-Benito et al. (2018) studied impact of the contrasting levels of DMA and NA on the Fe and Zn cation distribution within the endosperm and embryo of rice seeds using different transgenic lines overexpressing nicotianamine synthase (*OsNAS1*) and/or barley nicotianamine amino transferase (*HvNAATb*). The authors found that the transgenic lines exhibit three different DMA/NA changes:

1. An enhanced NA level (caused by overexpression of *OsNAS1*) was not fully decreased because of limited capacity to exploit NA for DMA synthesis (as a result of low expression of *HvNAATb*), and caused consistent enrichments in NA, DMA, Fe and Zn in the endosperm, and NA, DMA and Fe in the embryo.
2. An enhanced NA level (through overexpression of *OsNAS1*), was decreased by an enhanced capacity to exploit NA for DMA synthesis (through enhanced expression of *HvNAATb*), and caused enrichment only for DMA and Fe, both in the embryo and endosperm.
3. The insufficient NA renovation and limited DMA synthesis despite of the improved capacity to exploit NA to DMA (through overexpression of *HvNAATb*), caused changes in DMA level, decreases in NA level, and moderate decreases in Fe in the embryo and endosperm.

Masuda et al. (2019) developed a new rice variety tolerant to Fe deficiency by introducing of *IDS3* genome fragment of barley along with *refre1/372* and *OsIRO2* genes called "IRI" lines. Their results showed that an enhanced tolerance was observed in *OsIRO2* introduced line at the early growth stage and in *refre1/372* introduced line at the late stage. They also mentioned that the introduction of a combination of genes (*OsIRT1* promoter-*refre1/372* and the *35S* promoter-*OsIRO2* "RI" lines) enhanced tolerance in all stages among the five types of cultivation method. Furthermore, they reported that newly developed IRI rice lines exhibited improved tolerance to Fe deficiency rather than non-transgenic ones and also rather than rice lines harboring single introduction of either genes which overexpressing *OsIRO2* or the *IDS3* fragment when grown in submerged calcareous soil. The authors also reported that the yields of IRI lines were ninefold higher than the non-transgenic plants.

6.3.3 Biofortification via Genetic Engineering for Selenium

Genetic engineering, which has been shown to enhance Se accumulation, tolerance, and volatilization by plants, has focused on S-related enzymes. Regarding the transgenic approaches, the *selenocysteine methyltransferase* gene of *Astragalus bisulcatus* was introduced into *Arabidopsis thaliana* to overexpress Se-methyl

selenocysteine and γ-glutamyl methyl selenocysteine in shoots (Sors et al. 2005a, b; Pilon-Smits and LeDuc 2009) and resulted in an increased accumulation of Se.

In a survey conducted by Zhang et al. (2014), the effect of *OSPT2* gene expression on Se content of rice grains was investigated. In this regard, wild and transgenic rice plants were planted in the field, and the content of grain Se was determined after harvesting. The results showed that Se content in rice grains of plants with *OSPT2* overexpression was significantly higher than that of wild type. Likewise, Se content was significantly lower than that of wild type in OSPT2RNAi lines grains (Ri-2 and Ri-5). In addition, these results showed that Se content in grain could be improved by increasing Se uptake. The authors mentioned that overexpression of *OSPT2* can significantly increase Se uptake and accumulation in shoots and roots, thus leading to a further increase in Se content in rice grain, which could be a potential strategy for breeding enriched rice varieties.

Additionally, Zhang et al. (2014) described that a rice mutant (*ltn1*) that has previously been shown to increase phosphate uptake exhibited higher selenium uptake than wild type in both time-dependent selenite uptake and concentration assays. Respiratory inhibitors, which significantly inhibited selenite uptake in wild and the "*ltn1*" mutant variants, indicated that selenite uptake was associated with H^+ and energy-dependent. Selenium uptake was highly enhanced under Pi starvation conditions, indicating that Pi transport is involved in selenite uptake. Genetic engineering as an additional technique for breeding along with functional genomic gene technology can significantly contribute to future Se biofortification researches (Poletti and Sautter 2005).

6.3.4 Challenges Toward Biotechnologically Biofortified Crops

A major problem of developing biofortified crops is the expense of research and regulatory acceptance, on account of extreme precautionary regulation of biotechnology origin crops. On the other hand, a successful biofortification procedure requires widespread adoption of the crops by consumers, and this consequences different important challenges (Powell 2007). Also, public acceptance is crucial, especially if the new trait changes remarkably the quality of the crop, such as color, taste, and dry matter content. Sufficient information programs will play important role in ensuring acceptance. Moreover, due to possibility of epigenetic silencing in transgenic plants, transgene cassette with duplicated or inverted repeats may not be stable and inherited after several generations (Rajeevkumar et al. 2015; Boonyaves et al. 2017). Hence, further investigations should be performed to reveal the stability of transgenes or different approaches to maintain multiple transgenes over multiple generations (De-Xian Kok et al. 2018).

7 Phytate and Bioavailability of Nutrients

An important compound to be stored in seed is phytate, a complex cation salt of phytic acid which is accounted approximately 75% of total phosphorus content in seeds (Suzuki et al. 2007; Raboy 2009). Phytic acid (PA) as an essential substance

for seed development and higher seedling vigor may positively act as anti-cancer agent, antioxidant, lowering chronic disease rates, and heart diseases (Bohn et al. 2008; Gemede 2014). However, PA acts as a potent chelator of cations to form phytate and often is considered as an anti-nutritional substance because it reduces bioavailability of the important micronutrients. Since rice is the major nutrient source for more than half of the world population and is a poor source of essential micronutrients, therefore biofortification of rice seed in terms of Zn and reducing the PA content concomitantly has been raised as a new strategy for increasing micronutrient bioavailability in rice. Furthermore, global effects of climate change, particularly increasing atmospheric CO_2 concentration, are expected to increase the PA content and decrease the concentrations of most micronutrients in rice seed (Perera et al. 2018).

The use of varieties with high rates of Zn and low phytate contents, selection for individual and advanced fixed lines with low phytate content, and integrating phytate phenotyping together with grain Zn in breeding programs will improve developing lines with high Zn and low phytate content. Recently, several mutants with low phytate content have been developed by mutation breeding and are considered as good resources as low-phytate donors in breeding efforts (Liu et al. 2007).

It has been revealed that reducing the expression of the *1D-myo- inositol 3-phosphate synthase* gene (*RINO1*) in developing rice seeds might lead to a remarkable reduction in the phytin content of the seeds, as the 1D-myo-inositol 3-phosphate synthase enzyme catalyses the first step of myo-inositol biosynthesis and causes phytic acid biosynthesis in seeds. In this regard, reduced PA content in seeds has been achieved by manipulating the expression of the rice *myo-inositol 3-phosphate synthase* gene so that, *RINO1* cDNA was transformed into rice plants in the antisense orientation under the control of the rice major storage protein glutelin *GluB-1* promoter (Kuwano et al. 2006) and the*18-kDa Oleosin* promoter (Kuwano et al. 2009) to suppress *RINO1* gene expression in seeds. Ali et al. (2013) mentioned that silencing the rice *myo-inositol 3-phosphate synthase* gene might lead to the harmful changes in important metabolic pathways utilizing myo-inositol, which is considered a substance important in metabolic pathways of different plants. Hence, to decrease PA content of the seeds, rice plants achieved by silencing the *IPK1* gene (which catalyzes the last step of phytic acid biosynthesis in rice) and applying the *Ole 18* promoter in RNAi-mediated approach. The resulted transgenic rice varieties possessed a remarkable reduction in seed PA levels without inhibiting seed germination or development.

8 Conclusions

The nutritional value of enriched rice grain is essential for reducing malnutrition of developing countries. To solve this problem, studies are being carried out aiming to enrich rice grain through different biofortification methods. Each method is important for specific mineral. In this sense, it is important to indicate significance of the

agronomic biofortification of rice with elements featuring high mobility in the soil such as Se which is easily taken up and accumulated in grains in a bioavailable form. Also, method of mineral application is crucial to enrich grain. For example, foliar spraying of Zn, if performed at the reproductive stage of rice, may be more efficient than soil application. In addition, concomitant application of different nutrient elements may exhibit synergist or antagonist impacts. For instance, Fe and Zn absorption may be negatively affected by elements such as P. However, in the case of Zn, the use of conventional breeding methods—which refer to availability of gene based markers applied by breeders to introduce specific alleles known to contribute to the enrichment of rice grain—is presented as the recommended method due to the high genetic diversity of grain Zn content in rice germplasm. Eventually, the use of transformation method, on account of low genetic variation for grain Fe content within cultivated rice germplasms, was mentioned as a successful option to produce grains rich in iron. Though rice cultivars with target levels of grain Fe, Zn, and Se content have been developed and released commercially, studies on integration of agronomic biofortification with conventional breeding and transgenic approaches for enhanced nutrients bioavailability and also stability of traits in different environments are still in progress.

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Involvement of Policymakers, Public Acceptance, and Commercialization of Nutritionally Enhanced and Genetically Modified Rice

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Abstract

The advent of recombinant DNA technology along with periodic advancement of molecular technologies has paved the path for development of genetically modified (GM) crops. Despite being considered superior in terms of nutritional attributes, disease resistance, and quality, GM crops are not accepted widely for human consumption. The factors that determine the acceptance of the GM crops includes consumer health safety issues, environmental safety, rigid regulatory frameworks, and reluctance of policymakers. This review focuses on the various protocols that govern the development of GM rice varieties, the role of policymakers for commercialization of these transgenics, and also the growing public awareness highlighting the benefits of consuming nutritionally enhanced GM rice.

Keywords

GMO crop · GMO labelling · Golden Rice · Consumers' acceptance · Regulation · Public perception

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1 Introduction

Food security means when "all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life. Food insecurity exists when people do not have adequate physical, social or economic access to food as defined above" (Challa et al. 2019). "GM foods refer to foods produced from genetically modified plants or animals" (Zhang et al. 2016). GMOs that are commercially available in the market are listed in Table 1.

2 Importance of GM Foods

The challenges associated with food security are expansion of population, decrease in arable land, and bottleneck of conventional and modern breeding (Zhang et al. 2016). The human population as per 2015 Statistics is 7.35 billion and is expected to increase at a rate of 1.24% per year. It was estimated that the population in 2030 would be around 8.5 billion and in 2050 would be 9.7 billion (Cheeseman 2016). In order to meet the food demand of the growing population, agriculture is one of the ways. An annual increase of 2.4% crop yield is required to meet the needs of growing population (Raiten and Combs 2019). But the amount of arable land available for agriculture is decreasing to 0.18 ha by 2050 (Dutta et al. 2018, 2019; Wani et al. 2017). Maximum food production can be achieved with the available arable land if there are best breeds for crops. In this connection, a best breed with superior traits as an agriculture input is required for maximum agriculture production. But to develop the best breed with superior traits is time-consuming. Therefore, genetic modification of an organism can help in developing GM foods that are superior in traits. GM foods can boost the crop yields and help in providing food security to the growing population.

3 Developing GM Foods

Genetic modification in biology is a technique that affects the genetic machinery of the entire living kingdoms (Oliver 2014). According to the WHO (World Health Organization), "Organisms (i.e. plants, animals or microorganisms) in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating and/or natural recombination" are termed as GMO. The above statement helps to distinguish the direct manipulation of genetic material from the age-old practice of genetic improvement in the stock of plants and animals by implementing selective breeding. With rDNA technology, genes from one organism can be transferred into another, mostly unrelated. Similarly, the FAO (Food and Agriculture Organization of the United Nations) and the European Commission define GMO as product "not occurring naturally by mating and/or natural recombination" (FAO

Table 1 GMOs in agricultural biotechnology

Tolerance/ resistance	Target crop	Genetically conferred trait	Year of commission	Company	Country
Approved GM crops for commercial cultivation					
Herbicide tolerance	Soybean (*Glycine max L.*)	Glyphosate herbicide tolerance conferred by expression of a glyphosate-tolerant form of the plant enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) isolated from the soil bacterium *Agrobacterium tumefaciens*, strain CP4	1995	Monsanto	USA, Argentina, Canada, Mexico, Romania, Uruguay, South Africa
	Maize (*Zea mays* L.)	*CEPSPS* gene (glyphosate herbicide resistance) from *Agrobacterium* C4, tolerance to glyphosate-based herbicides	1996/1997	Monsanto, Mahyco	France, Spain, Germany, North America, UK, Canada
	Canola (*Brassica napus* var. *napus* L.)	EPSPS gene (glyphosate herbicide resistance) and bromoxynil herbicide resistance	1996	Monsanto, AgrEvo	Canada, France, Japan, USA, UK
	Flax (*Linum usitatissimum* L.)	Tolerant to soil residues of sulfonylurea herbicide	1999	University of Saskatchewan	Canada, Brazil, EU countries
	Sugar beet (*Beta vulgaris* L.)	Herbicide resistance to Glufosinate HR Glyphosate HR	1998 1998	AgrEvo Novartis and Monsanto	USA, Canada
Insect/pest resistance	Potato (*Solanum tuberosum* L.)	*Bt* gene (*cry3A*) is a viral helicase leads to Colorado potato beetle insect and potato leaf roll virus resistance	1996–1999	Alpha, Norteña, Rosita	Netherlands, Mexico, Germany, Mediterranean countries
	Maize (*Zea mays* L.)	*Bt* gene resistant to corn borer pest and corn root worm	1995, 2003	Northrup King (Sandoz) Monsanto	France, Spain, Germany, North America, UK, Canada
	Cotton (*Gossypium hirsutum* L.)	A vector construct of *cry1A* and *cry2A* gene of *B. thuringiensis.* Transgenics are resistant *to* lepidopteran insects	1995	Monsanto, Mahyco, Syngenta	USA, Argentina, Canada, China, India Mexico, Brazil, Kenya

(continued)

Table 1 (continued)

Tolerance/ resistance	Target crop	Genetically conferred trait	Year of commission	Company	Country
	Tomato (*Solanum lycopersicum*)	Increased shelf life due to reduced ethylene Truncated ACC synthase gene delayed ripening *Bt* (cry1Ac) gene resistant to lepidopteran insects	1992 1995 1998	Calgene Zeneca and Petoseed Monsanto	Argentina, Brazil, China, Mexico
Virus resistance	Squash (*Curcubita pepo* L.)	Viral coat protein gene insertion (CP) gene imparting resistance to zucchini Yellow VR, watermelon mosaic VR, cucumber mosaic VR	1994 1994 1996	Upjohn Upjohn Asgrow	USA, Hawaii
	Papaya (*Carica papaya* L.)	Viral coat protein gene resistance to papaya ringspot virus	1998	SunUp, UH Rainbow	USA, New Zealand

Modified from https://m.jagranjosh.com/general-knowledge/ and AGBIOS database. Available online at: http://www.agbios.com

2016). "GM foods" hence are foods produced from genetically modified plants or animals.

However, Oliver (2014) pointed out the aforementioned definitions are somewhat incorrect by citing the example of triticale, a crop species widely used in bread and pasta that is developed in the nineteenth century by crossbreeding wheat and rye. However, the resulting hybrid was sterile, and in the 1930s, the chemical colchicine was used to generate polyploidy embryo cells, which were fertile. Triticale thus seems to unambiguously fit the definition of a GMO, even if the genetic modification is somewhat primitive by recent molecular biology techniques. Thus, Oliver suggested "biotechnologically modified organism" as a closer definition for GMO.

3.1 History of GM Foods

The initiation of DNA modification technologies dates back to 1944, when scientists discovered that genetic material can be transferred between and among different species (Avery et al. 1944). In 1954, Watson and Crick discovered the double helix structure of DNA and the "central dogma" of DNA. Nobel Laureate Marshall Nirenberg and coauthors (1963) and others had deciphered the genetic code in 1963. In 1973, Cohen et al. (1973) developed rDNA technology, thus showing that transfer of genetically engineered DNA molecules was possible among different species. The history DNA transfer actually begins with Charles Darwin's notions of species variation and selection. Table 2 presents a sort of time capsule of the seminal discoveries that are crucial to modern genomics.

The first two plants to be genetically modified were antibiotic-resistant tobacco and petunias in 1983 (Bevan and Chilton 1982; Fraley 1983). Scientists in China first commercialized genetically modified tobacco in the early 1990s. In 1994, the US market saw the first genetically modified species of tomato with the property of delayed ripening approved by the Food and Drug Administration (FDA). Since then, several transgenic crops have received FDA approvals, including "canola" with modified oil composition, cotton and soybeans resistant to herbicides, etc. GM foods that are available in the market include rice, potatoes, eggplants, strawberries, and carrots, and many more are still in the pipeline (Bawa and Anilakumar 2013).

GM crops help reduce malnutrition by enhancing yield, nutritional quality, and resistance to major biotic and abiotic stresses. However, several biosafety issues and public concerns are associated with cultivation of GM crops developed by transgenesis, i.e., introduction of genes from distantly related organism. Recent studies helped in developing alternative concepts of cis-genesis and intra-genesis. These involve transformation of plants with genetic material derived from the same species, which are closely related and capable of sexual hybridization, respectively. rDNA technology that targets site-specific integration of modified gene is found to be better than the traditional genetic engineering methods based on random integration of numerous copies of the transgene into plant genome which ultimately leads to gene silencing and unpredictable expression pattern of the transgenes.

Table 2 Time events in the history of genetic modification

S. No.	Year	Events
1	1859	Charles Darwin published the first edition of *On the Origin of Species*
2	1865	Gregor Mendel discovered that heredity transmitted in units
3	1869	Friedrich Miescher isolated DNA
4	1902	Walter Sutton developed chromosome theory of inheritance
5	1911	Thomas Hunt Morgan showed chromosomes carry genes
6	1941	George Beadle and Edward Tatum hypothesized one gene one enzyme theory
7	1944	Oswald Avery et al. demonstrated DNA can transform the properties of cells
8	1952	Alfred Hershey and Martha Chase showed that genes are made of DNA
9	1953	Francis H. Crick and James D. Watson described the double helix structure of DNA
10	1958	Matthew Meselson and Franklin Stahl discovered the semiconservative replication of DNA
11	1961	Sydney Brenner et al. reported that mRNA ferries information from DNA
12	1966	Marshall Nirenberg et al. cracked genetic codes
13	1968	Steward Linn and Werner Arber described first restriction enzyme
14	1973	Stanley Cohen and Herbert Boyer invented DNA cloning
15	1977	Richard Roberts and Phil Sharp discovered introns
16	1980	Jon W. Gordon et al. made first transgenic mice
17	1983	Kary Mullis invented PCR (polymerase chain reaction)
18	1985	Generate the first transgenic domestic animal, a pig
19	1987	First human genetic map was discovered
20	1990	Human genome project was launched
21	1991	First gene therapy trials on humans
22	1992	The second-generation genetic map of human genome was developed
23	1993	FDA approved the use of bovine somatotropin (bST) to increase milk production in dairy cows
24	1994	FDA approved the sale of the first GM food, the FLAVR SAVR tomato
25	1996	The birth of Dolly the sheep, the first cloned animal
26	1997	The *E. coli* genome was sequenced
27	1998	*M. tuberculosis* bacterium and roundworm *C. elegans* were sequenced
28	1999	The first human chromosome, chromosome 22, was decoded
29	2002	Mouse genome working draft was assembled
30	2003	The human genome sequencing was completed
31	2004	First field trial of Golden Rice cultivar

Source: https://www.gmeducation.org/faqs/p14924820brief%20history%20of%20genetic%20modification.html and https://www.genome.gov/Pages/Education/GeneticTimeline.pdf

Genome editing, which involves the use of engineered nucleases, allows the alterations or mutations of our gene of interest without using any foreign DNA. As a result, the plants developed can be considered as non-transgenic but genetically altered plants. This new technique would pave way for the development and commercialization of transgenic plants with superior phenotypes even in countries where GM crops are poorly accepted (Kamthan et al. 2016).

4 Benefits of GM Foods

4.1 Insect Resistance

Some GMO foods have been modified to make them more resistant to insects and other pests. A report from the University of California in San Diego states that toxic bacteria (yet safe for human use) can be added to crops to make them repel insects. Thus, the amount of pesticide and their exposure on the plants are limited (Brookes and Barfoot 2014).

4.2 Superior Crops

Another benefit of GM technology is that crops can be engineered to withstand extreme weather, indicating good quality and sufficient yields even under severe weather condition. As global population increases, more lands are being utilized for housing instead of food production, thereby prompting farmers to grow crops in locations that are originally not suitable for plant cultivation, and culturing plants that can withstand high salt content in soil and groundwater, not to mention long periods of drought, will help them grow healthy crops. Also, animals and plants that have been genetically modified can become more resistant to unexpected diseases (Brookes and Barfoot 2014; James 2013).

4.3 Environmental Protection

Increase of GM animals and crops often requires less time, tools, and chemicals. They may help in reducing greenhouse gas emissions, soil erosion, and environmental pollution. This means the general health and beauty of the environment surrounding farms will be improved, contributing to the preservation of better water and air quality, which can also indirectly benefit every person's well-being (Rizzi et al. 2012).

4.4 Improved Nutrition and Shelf Life

GM foods have been engineered to become more nutritious in terms of vitamin or mineral content. This not only helps people get the nutrients they need but also plays a significant role in fighting against malnutrition in Third World countries. GM foods were created with the use of genetic engineering, a technology that was designed to make sure crops will never be damaged in a fast rate. The method allows farmers and merchants to preserve the shelf life of foods more efficiently by using special substances (Schmidt et al. 2008; Moretti et al. 2014).

4.5 Decreased Use of Pesticides

It has been proven that genetically modified crops do not need pesticides to become stronger against various types of insects or pests that may destroy those (Kramkowska et al. 2013).

4.6 More Income

With genetic engineering, farmers will have more income, which they could spend on their own welfare, such as education of their children and medical welfare (Nicolia et al. 2014; Kramkowska et al. 2013).

4.7 Less Deforestation

To sufficiently feed the growing population of the world, deforestation is needed. But with genetically modified animals and crops, the use of deforestation will be minimized. This would decrease carbon dioxide in the atmosphere, which would, in turn, slow global warming (Nicolia et al. 2014).

4.8 Decrease in Global Warming

As more plants and crops can be grown, including those that were previously unsuitable for farming, oxygen in the environment is increased. This eventually decreases the proportion of carbon dioxide, thereby, in turn, reducing global warming. In fact, British economists noted in a study that genetically modified crops have made significant contribution in reducing greenhouse gas emissions by over 10 million tons, which is equivalent to removing 5 million cars from the road each year. This means that people would not have to give up their vehicles (Ellstrand and Hancock 1999; Chandler and Dunwell 2008).

4.9 Decrease in Food Prices

Due to higher yield and lower costs, food prices would go down as people in poorer countries spend over half of their income on food alone, it implies automatic reduction of poverty (Kramkowska et al. 2013).

4.10 New Products

New kinds of crops are being developed to be grown at extreme climates, such as those present in dry or freezing environments. As an example, scientists have

developed a new type of tomato that grows in salty soil. Another good discovery in genetic engineering of plants is the exclusion of the gene responsible for caffeine in coffee beans, creating decaffeinated coffee beans, which can then be grown naturally (Chandler and Dunwell 2008).

5 Potential Risks of GM Foods

5.1 Allergic Reactions

According to research by the Brown University, recent genetically modified foods can pose significant allergy risks to people. It states that genetic modification often adds or mixes proteins that were not indigenous to the original animal or plant, which might cause new allergic reactions in our body. In some cases, proteins from organisms that you are allergic to might be added to organisms that you were not originally allergic to; thereby, your range of food choices will be lessened.

5.2 Lower Level of Biodiversity

One big potential drawback of the technology is that some organisms in the ecosystem could be harmed, which in turn could lead to a lower level of biodiversity. When we remove a certain pest that is harmful to crops, we could also be removing a food source for a certain species. In addition, genetically modified crops could prove toxic to some organisms, which can lead to their reduced numbers or even extinction.

5.3 Decreased Antibiotic Efficacy

According to the Iowa State University, some genetically modified foods have antibiotic features that are built into them, making them resistant or immune to viruses or diseases. So when we eat them, these antibiotic markers will persist in our body and will render actual antibiotic medications less effective. The university also warns that ingestion of such foods and regular exposure to antibiotics may contribute to the reduced effectiveness of antibiotic drugs, as noticed in hospitals across the planet (Baulcombe et al. 2014).

5.4 Cross-Pollination

Cross-pollination can cover quite large distances, where new genes can be included in the offspring of organic, traditional plants or crops that are miles away. This can result in difficulty in distinguishing which crop fields are organic and which are not, posing a problem to the task of properly labelling non-GMO food products.

5.5 Gene Spilling

It is unclear what effects, if there are any, the genetic pollution resulting from inadequate sequestering of genetically modified crop populations would have on the wild varieties surrounding them. However, it is stressed that releasing pollen from genetically altered plants into the wild through the insects and the wind could have dramatic effects on the ecosystem, though there is yet long-term research to be done to gauge such impact (Snow and Palma 1997).

5.6 Gene Transfer

Relevant to the previous disadvantage, a constant risk of genetically modified foods is that an organism's modified genes may escape into the wild. Experts warn that genes from commercial crops that are resistant to herbicides may cross into the wild weed population, thus creating super-weeds that have become impossible to kill. For genetically enhanced vegetation and animals, they may become superorganisms and can potentially outcompete natural plants and animals, driving them into extinction (Gilbert 2013).

5.7 Widening Gap of Corporate Sizes

This disadvantage can possibly happen between food-producing giants and their smaller counterparts, causing a consolidation in the market. There would be fewer competitors, which could increase the risk of oligopolies and food price increases. Moreover, larger companies might have more political power and might be able to influence safety and health standards.

5.8 New Diseases

As previously mentioned, genetically modified foods can create new diseases. Considering that they are modified using viruses and bacteria, there is a fear that this will certainly happen. This threat to human health is a worrisome aspect that has received a great deal of debate.

5.9 Food Supply at Risk

GMO seeds are patented products, and, in order to purchase them, customers have to sign certain agreements for use with the supplier or creator. As the reliance on these seeds expands around the world, concerns about food supply and safety also continue to arise. Furthermore, these seeds are structurally identical, and if a problem affects one of them, a major crop failure can occur (Aggarwal 2012).

5.10 Economic Concerns

Bringing a genetically modified food to market can be a costly and lengthy process, and of course, agricultural biotechnology companies want to ensure a profitable ROI (return on investment). So many new plant genetic engineering technologies and products have been patented, and patent infringement is a big concern within the agribusiness. Also, consumer advocates are worried that this will increase seed prices to very high levels beyond the reach of Third World countries and small farmers, thus widening the gap between the rich and the poor.

6 Rice as GM Food

6.1 Nutritionally Enhanced GM Rice

Plants or crops grown worldwide as food offer a wide range of bioactive compounds that are of immense importance to the overall human health. The availability of high nutritional content food crops is limited for those who live in the industrialized world; however, this is not always the case for the rural poor who reside in developing countries. For such Third World populations, a balanced diet that is adequate in optimum levels of vitamins and minerals can be difficult to achieve and maintain (Gilani and Nasim 2007). More so often, a monotonous diet predominated by a single crop such as rice is all that is on hand and affordable. Fortunately, due to recent developments in agricultural biotechnology, it is now possible to generate food crops that are nutritionally enhanced to improve the content and bioavailability of essential nutrients, such as iron and vitamin A (Pérez-Massot et al. 2013; Farre et al. 2011; Pachon et al. 2009). Technologies with similar efficacy have been used to ward off chronic illnesses including heart disease and cancer (Glen 2008).

Rice has also been engineered to combat other major forms of malnutrition, including iron and folate deficiency (Vasconcelos et al. 2003). These were addressed by improving iron storage and transport proteins in plants and by adding a phytase to improve ion absorption in the gut (Cockell 2007; Landoni et al. 2013). Transgenic rice that expresses essential amino acids such as free lysine has also been developed using RNAi silencing-based technologies. De Steur et al. (2012) demonstrated that transgenic biofortified rice could be cost-effective in alleviating folate deficiency rather than conventional supplementation programs. Iron has been increased in rice as a result of conventional plant breeding rather than the development of transgenic plants. Haas et al. (2005) demonstrated that Filipino women consumed 1.79 mg iron/day in the form of steamed biofortified conventional rice. A control group consumed non-biofortified rice at a level of 0.37 mg of iron/day. Studies using transgenic rice that have been biofortified with iron have centered on the overexpression of iron storage proteins such as ferritin. Rice cultivated from these transgenic plants contain three to four times as much iron as their wild-type counterparts (Lucca et al. 2002).

6.1.1 Golden Rice

Each year, vitamin A deficiency causes eye damage in three million preschool-aged children. Of these, half a million become blind and two-thirds will die shortly afterward. The precursor molecule required for vitamin A biosynthesis, β-carotene, is absent from the grain of cereals such as rice. As a result, many with a largely monotonous diet are at risk of vitamin A deficiency. Golden Rice, named for its golden color due to its high β-carotene content and generated using biotechnology, was designed by the research group of Ingo Potyrus and offers a viable solution. The transgenic crop was engineered with two genes from other organisms (daffodil and the bacterium *Erwinia uredovora*) which reconstitute the carotenoid biosynthetic pathway within the rice genome (Fig. 1; Tang et al. 2009a, b). This new trait was then transferred into high-yielding local commercial cultivars by marker-assisted backcross breeding.

The most current Golden Rice technology, known as GR2, utilizes genes from two distinct provitamin A pathways, including the substitution of the phytoene synthesis gene from maize for the analogous daffodil gene used in GR1 rice (Tang et al. 2012; Shumskaya and Wurtzel 2013). Golden Rice can produce levels of β-carotene that were up to 35 μg/g of dry rice (Tanumihardjo et al. 2010). Servings of 130–200 g of deuterium-labelled Golden Rice grown hydroponically in heavy water expressing 0.99–1.53 mg β-carotene were fed to human volunteers. Blood samples taken at 36th days exhibited 0.34–0.94 μg retinol, indicating that β-carotene derived from Golden Rice is effectively converted to vitamin A at a rate of 500–800 μg retinol per 100 g uncooked Golden Rice, close to the recommended daily allowance for children (Tanumihardjo et al. 2010).

The vitamin A content of Golden Rice, non-transformed spinach and β-carotene provided in oil to children were compared. The results showed that the β-carotene derived from Golden Rice was just as effective as pure β-carotene and was more effective than β-carotene obtained from spinach in providing vitamin A to children (Haskell 2012). Together, these results suggest that Golden Rice could realistically be used to alleviate vitamin A deficiency in rice-consuming populations (Xudong et al. 2000). Golden Rice could be considered the very first genetically engineered crop that was specifically designed to combat malnutrition (WHO 2017). The selective advantage of a biofortified crop such as Golden Rice is that it could readily reach remote rural populations which have no access to supplementation programs (Moghissi et al. 2015; Bouwman et al. 2014).

"Golden Rice" is the first purposefully created biofortified food. It synthesizes and accumulates β-carotene during seed maturation (Ye 2000). Following normal harvesting, grain polishing, storage, cooking, and consumption, the human body efficiently converts the β-carotene in Golden Rice into vitamin A (Tang et al. 2009a, b). "In summary, the high bioconversion efficiency of Golden Rice beta-carotene to vitamin A shows that the transgenic rice variety can be used as a source of vitamin A. Golden Rice may be as useful as a source of preformed vitamin A from vitamin A capsules, eggs or milk to overcome VAD in rice-consuming populations" (Dubock 2013; Stokstad 2015), "so that a few ounces of cooked rice can provide enough to eliminate the morbidity and mortality of Golden Rice" (Fedoroff 2015).

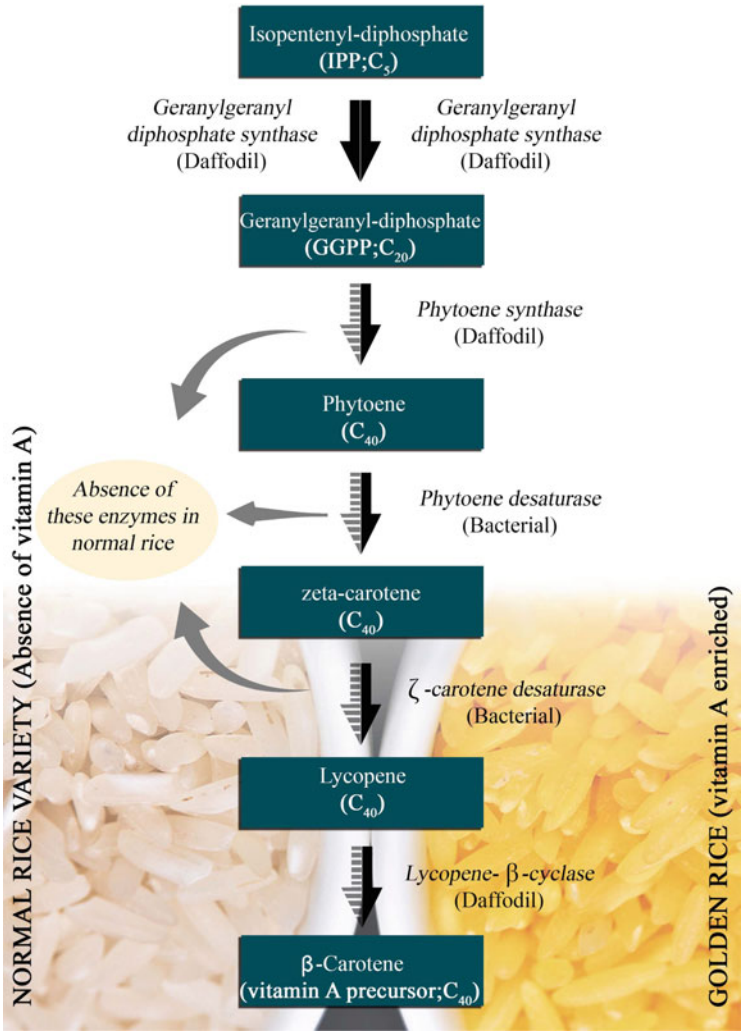

Fig. 1 Biosynthetic pathway of Golden Rice

Reports suggest that 40 g of dry Golden Rice, after normal harvest, polishing, storage, and cooking, when consumed daily, will save life and sight of people who would otherwise be vitamin A-deficient (Dubock 2013).

6.2 Other Traits Enhanced GM Rice

6.2.1 Herbicide Resistance

Weeds are a constant problem in rice fields. Weeds compete with the crops for water, nutrients, sunlight, and space. They also harbor insects and diseases, clog irrigation and drainage systems, undermine crop quality, and deposit weed seeds into crop harvests. If left uncontrolled, weeds can reduce crop yields significantly. The tandem technique of soil tilling and herbicide application is an example of how farmers control weeds in their farms (Brookes and Barfoot 2018).

Researchers postulated that weed management could be simplified by spraying a single broad-spectrum herbicide over the field anytime during the growing season. However, recent advancements in GE, herbicide-tolerant (HT) rice crops offer farmers a vital tool in fighting weeds and also help preserve topsoil. These give farmers the flexibility to apply herbicides only when needed, to control total input of herbicides, and to use herbicides with preferred environmental characteristics (Carpenter and Gianessi 2001; Carpenter et al. 2002).

These herbicides target key enzymes in the plant metabolic pathway, which disrupt plant food production and eventually kill it. Some plants acquire the trait through selection or mutation; or more recently, plants are modified through genetic engineering. Other methods by which crops are genetically modified to survive exposure to herbicides include (1) producing a new protein that detoxifies the herbicide, (2) modifying the herbicide's target protein so that it will not be affected by the herbicide, or (3) producing physical or physiological barriers preventing the entry of the herbicide into the plant. The first two approaches are the most common ways scientists develop herbicide-tolerant crops. There is only a single report from the public sector, to the best of our knowledge, mentioning the development of PGMS (photoperiod-sensitive genic male sterile) transgenic rice which was engineered with dual herbicide tolerance traits against the glyphosate and glufosinate herbicides (Deng et al. 2014).

6.2.2 Pest Resistance

The economic benefits of insect-resistant genetically modified (GM) crops are now well known, but the positive impact of such crops and the consequent reduction in pesticide use on farmers' health remains largely unknown. Various studies and its subsequent analysis revealed that GM rice significantly reduces pesticide use and the resultant adverse effects on farmers' neurological, hematological, and electrolyte system (Zhang et al. 2016). Other benefits include increased yield and revenue from crop cultivation. Hence, the commercialization of GM rice is expected to improve the health of farmers in developing countries, where pesticide application is necessary to mitigate crop loss (Huang et al. 2005).

The most famous example is BT rice which was modified to express the cryIAb gene of the *Bacillus thuringiensis* bacterium (Fujimoto et al. 1993). It exerts resistance to various pests like the rice borer by producing endotoxins. Currently, field trials on insect-resistant cultivars are active in China. The trait helps the farmers as they avoid spraying their crops with pesticides to control fungal, viral, or bacterial

pathogens, which otherwise they would have done three to four times every season. BT rice was approved in China for large-scale cultivation from 2009 onward (James 2009). The BT rice varieties were found to be resistant against striped stem borer, leaf folder, and yellow stem borer (Bakshi and Dewan 2013). Since 1989, China has been actively involved in the development of insect resistance genetically modified (IRGM) transgenic rice varieties with various insecticidal genes (cry1Aa, cry1Ab, cry1Ac, cry1C, cry2A, CpTI [cowpea trypsin inhibitor], etc.) exhibiting high lepidopteran activity (Table 3).

Several viral diseases have also been targeted via RNAi-mediated resistance. It was observed transgenic rice harboring Psn12 and Psn4 (nonstructural proteins in insects) RNAi cascade conferred resistance to rice dwarf virus (RDV) infection by accumulating specific si-RNAs (Shimizu et al. 2009).

6.2.3 Allergy Resistance

Certain rice seed proteins are capable of inducing food allergy in patients with clinical symptoms ranging from eczema to dermatitis. Wakasa et al. (2011) reported that serum of allergy patients contained high levels of α-amylase/trypsin inhibitors and β-glyoxalase. These potential allergens were identified based on their unique recognition by serum IgE. Researchers mainly in Japan are developing hypoallergenic rice cultivars to repress the formation of allergen AS-Albumin (Dutta et al. 2016). These researchers tested GM rice on macaque monkeys that prevent allergies to cedar pollen, otherwise responsible for causing hay fever, accompanied with itchy eyes, sneezing, and other serious allergic reactions. The modified rice had seven proteins within the cedar pollen that prevent these allergic reactions (Coghlan 2009). Currently, human clinical trials with this particular rice transgenic are actively going on.

Chinese scientists modified brown rice as a cost-effective way to produce HSA protein (human serum albumin), which is a blood protein in human blood plasma. Chinese scientists put recombinant HSA protein promoters into 25 rice plants using *Agrobacterium*-mediated gene transfer. Out of the 25 plants, 9 contained HSA protein. The genetically modified brown rice had the same amino acid sequence as HSA. They called this protein *Oryza sativa* recombinant HSA. The modified rice was transparent (Coghlan 2009) and can be used for treating severe burns, liver cirrhosis, and hemorrhagic shock.

6.2.4 C4 Photosynthesis

Rice, a C3 crop, is a staple food for more than half of the world's population, with most consumers living in developing countries. Engineering C4 photosynthetic traits into rice is increasingly suggested as a way to meet the 50% yield increase that is predicted to be needed by 2050. Advances in genome-wide deep sequencing, gene discovery, and genome editing platforms have brought the possibility of engineering a C3 to C4 conversion closer than ever before. Because C4 plants have evolved independently multiple times from C3 origins, it is probably that key genes and gene regulatory networks involved in regulation of C4 were recruited from C3 ancestors.

Table 3 List of GM rice approved for field trials and commercial cultivation

S. No.	GM trait	Foreign gene introduced	Biological function	Developing company
1	Anti-allergy	7crp	Induces immune tolerance to cedar pollen	NIAS (Japan)
	Antibiotic resistance	aph4 (hpt)	Selection for resistance to hygromycin B antibiotic	
2	Lepidopteran insect resistance (BT Shanyou 63 and Huahui-1 variety)	cry1Ab	Induces tolerance to lepidopteran insects by distorting their midgut lining	Huazhong Agricultural University (China)
		cry1Ac	Induces tolerance to lepidopteran insects by distorting their midgut lining	
3	Glufosinate herbicide tolerance	*Bar*	Induces glufosinate tolerance via acetylation	Bayer Crop Science
	Lepidopteran insect resistance	aph4 (hpt)	Selection for resistance to hygromycin B antibiotic	
	Antibiotic resistance	*cry1Ab* (truncated)	Induces tolerance to lepidopteran insects by distorting their midgut lining	
4	Insect resistance	cry2	Induces tolerance to lepidopteran insects	Indian Agricultural Research Institute, Shillong, Meghalaya
5	Insect resistance	cry1Ab	Induces tolerance to lepidopteran insects	Maharashtra Hybrid Seeds Co. Ltd. (Mahyco)
6	Insect resistance	cry1Ab cry1Ac cry1Ab	Induces tolerance to lepidopteran insects by distorting their midgut lining	Metahelix
7	Drought and salinity tolerance	B6 and C15/gly I and gly II	Induces drought and salt tolerance via glyoxalase pathway	Bioseed Research, India
8	Nutritional enhancement	ferritin gene	Overexpression of ferritin gene increases iron production	BASF Department of Botany, University College of Science, University of Calcutta

Modified from *ISAAA Brief 46-2013* and GEAC meetings minute, http://www.geacindia.gov.in

In their study, researchers introduced a single maize gene to the rice plant to make it more efficient at photosynthesis (Miyao 2003; Agarie et al. 2002).

Rice normally uses a photosynthetic pathway called C3, which in hot and dry environments is much less efficient than the C4 pathway used by other plants (Bullis 2015). But if rice could be "switched" to use C4 photosynthesis, it could increase productivity by 50%. Despite only being used by 3% of plant species, the C4 pathway accounts for around a quarter of productivity on Earth. To achieve the target, a single maize gene called GOLDEN2-LIKE was introduced to the rice plant. This increased the volume of chloroplasts (structures where photosynthesis takes place) and mitochondria (structures that provide energy) in the sheath cells surrounding leaf veins.

7 Regulatory Authorities for GM Food and Rice

7.1 Regulation in India

India endorsed the Cartagena Protocol on Biosafety and constituted institutions such as the Biosafety Clearing House (MoEF&CC 2017) to regulate biosafety issues related to GM crops. Cartagena Protocol on Biosafety governs the biosafety regulations by outlining the essential requisites related to handling of living modified organisms (LMOs), commercialization, and deregulation of GM crops. Regulation of GM crops and products began in 1982 and was supervised by the National Biotechnology Board (NBB) based on stringent biosafety guidelines (Chaturvedi 2004). In 1986, the NBB was transformed into the Department of Biotechnology (DBT) and is governed by the Ministry of Science and Technology (Sharma 2005). The Ministry of Environment, Forest and Climate Change (MoEF&CC) is another regulatory body that partakes in regulation of GMOs and products developed from them by the Environmental Protection Act 1986 (EPA 1986) (Kolady and Herring 2014).

The above two regulatory constitutions appraise and safeguard the biosafety of GM crops, products, and research by the coordinated supervision of six proficient organizations, namely, the Recombinant DNA Advisory Committee (RDAC), the Review Committee on Genetic Manipulation (RCGM), the Genetic Engineering Appraisal Committee (GEAC), the Institutional Biosafety Committees (IBSC), the State Biotechnology Coordination Committee (SBCC), and the District Level Committees (DLC) (Shukla et al. 2018; MoEF and BCIL, New Delhi 2015).

The GEAC is constituted under MoEF&CC and functions in accordance with the "Rules for Manufacture, Use, Import, Export, and Storage of Hazardous Microorganisms/Genetically Engineered Organisms or Cells 1989," which comes under the provision of EPA, 1986. It is involved in the review of GM crops such as GM rice, and approval is based on the impact of such crops on the environment on its release and during field trials.

GOI also established Biotechnology Regulatory Authority of India (BRAI) aimed toward fast tracking of the regulatory assessment procedures of potential GM and

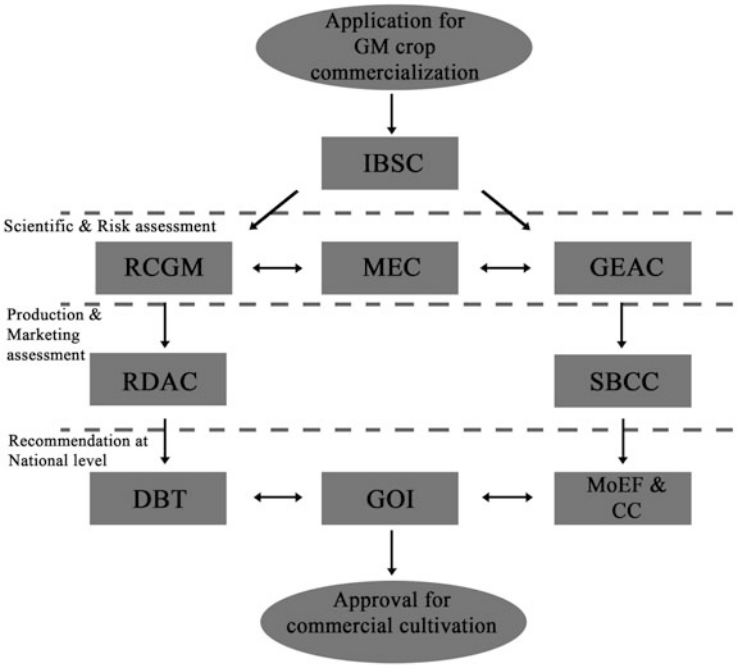

Fig. 2 Regulatory framework for approval of GM crop commercialization in India

biotechnology products to allow their quick access to our farmers (PIB, Government of India 2011). In 2013, the BRAI bill was first introduced in Lok Sabha and has subsequently undergone several revisions (PRS Legislative Research 2013). Under this regulatory organization, designated scientific experts are entitled to conduct multilevel assessment on important aspects related to GM crops and products such as research, transport, containment, release in environment, and their production.

The Indian Council of Agricultural Research (ICAR) in collaboration with several national agricultural research institutes developed the Indian Hybrid Rice Program in 1989 (Warrier and Pande 2016). Despite limited resources in terms of germplasm, inferior grain quality, and availability of hybrid seeds, the ICAR has been instrumental in the approval of several GM rice in different stages of field trials (Fig. 2).

7.2 Regulation in China

China was one among the first few countries to venture into commercialization of GM crops and products. It is ranked sixth in the world in terms of area under commercial plantation (James 2016). Till date, Bt cotton (1996) and ring spot virus-resistant papaya are allowed for commercial cultivation, and later, seven

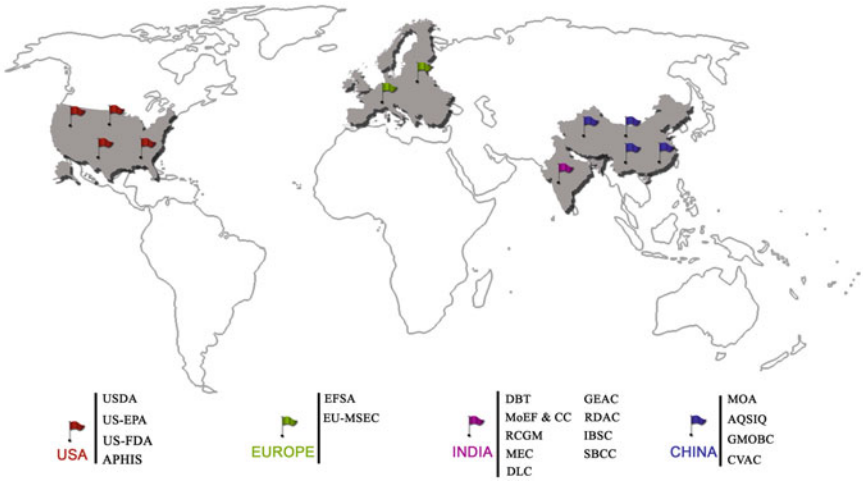

Fig. 3 List of regulatory authorities involved in commercialization of GM crops in the USA, Europe, India, and China. *USDA* United States Department of Agriculture, *US-EPA* United States Environmental Protection Agency, *US-FDA* United States Food and Drug Administration, *EFSA* European Food Safety Authority, *EU-MSEC* European Union Member State European Committee, *DBT* Department of Biotechnology, *MoEF&CC* Ministry of Environment, Forest and Climate Change, *RCGM* Review Committee on Genetic Manipulation, *MEC* Monitoring cum Evaluation Committee, *DLC* District Level Committees, *GEAC* Genetic Engineering Appraisal Committee, *RDAC* Recombinant DNA Advisory Committee, *IBSC* Institutional Bio-safety Committees, *SBCC* State Biotechnology Co-ordination Committees

other GM crops (papaya, petunia, sweet pepper, tomato, rice, cotton, and corn) for field trials were approved (Lu 2010; Li et al. 2014).

China is actively involved in the production of GM rice, and so far, results are encouraging at the preproduction and field trials. Insect-resistant Bt rice cultivars recorded high grain yield and significantly low pesticide input. Low pesticide use was shown to improve the health of farmers who were previously exposed to the toxic effects of pesticides (Huang et al. 2005) The MOA and Administration of Quality Supervision, Inspection and Quarantine (AQSIQ) are the regulatory bodies who oversee the policy and safety assessment, GMO labelling, and regulation of GM crops and products (Wong and Chan 2016). The regulatory authorities involved in commercialization of GM crops in India, the USA, China, and Europe are highlighted in Fig. 3.

Approval from MOA is mandatory for agricultural GMOs during testing, production, processing, import, and marketing stages of its commercialization (MOA 2015). The companies or organizations involved in production and marketing of GM products need to acquire a biosafety certificate, crop variety certificate, production license, and marketing license. The biosafety certificate has to be obtained from the GMO Biosafety Committee and is only given after successfully meeting the safety assessment standards. It is valid for a period of five years and requires renewal after every five years (Jia and Peng 2002). The biosafety certificate is mandatory for

transgenic organisms (seeds, livestock, and poultry breeds) and the products developed using agricultural GMO components (pesticides, veterinary drugs and fertilizers). Commercialization of any GM crop or products needs to require clearance at each of the five stages, namely, laboratory research, restricted field trials, environmental release field trials, and preproduction testing to successfully obtain a biosafety certificate. The MOA classifies agricultural GMOs into five categories (Class I, II, III, or IV) based on the risk element associated with any other life-forms (human, animals, plants, etc.) and the environment (MOA 2015). Till date, 2775 field trials for GM crops, 459 environment release field trials, and 317 preproduction testing applications have been consented by the MOA from 2002. The crop variety is essential for any new variety of agricultural crops (rice, wheat, corn, cotton, soybean, canola, and potatoes) and the samples are analyzed by the Crop Variety Approval Committee under the regulation of the National Seed Law.

Labelling of GM crops, foods, and products is stringently monitored, and all products in sale should be appropriately labelled under the "Safe and Accurate Food Labeling Act of 2015." At present, GMO labelling is mandatory for transgenic seeds (cotton, soybean, corn, canola, and tomato) and products derived from such transgenic crops (soybean oil, corn flour, and tomato paste) (Zhang et al. 2018).

AQSIQ is involved in the inspection of import and export of agricultural GMO products and functions in accordance with the AQSIQ regulation. Such products on import are subjected to standard genetic tests to confirm their transgenic nature and identify the individual GM components (Jin et al. 2019). If products do not match the required standards, they are either ceased or destroyed (Qiu 2014; Wong and Chan 2016).

Rice being a staple food for majority of the Chinese population, the government has funded several GM rice projects. So far, only two rice varieties, "Bt Shanyou 63" and "Huahui No. 1," have obtained biosafety certificate for commercial cultivation in its native province, Hubei, but both are yet to obtain the crop variety certificate essential for its sale (Li et al. 2016).

7.3 Regulation in the US

Since 2010, the USA has significantly contributed to the cultivation of transgenic crops, and at present, GM alfalfa, canola, corn, cotton, papaya, soybean, sugar beets, and squash are being cultivated for commercial use (Parisi et al. 2016). So far, the USA has approved more than 15,000 restricted field trials of several transgenic plants (tomato, tobacco, soybean, cotton, cucumber, poplar, potato, alfalfa, squash, walnut, melon, rice, canola, corn, etc.). Transgenic crops such as corn and canola are used for the production of animal feed and cater 95% of animal livestock in the USA. There is no discrete federal law pertaining to agricultural GMOs or products derived from them. However, the US legal system permits a state to constitute their own laws to govern GM crops or foods without the interference of federal law (Yang and Chen 2016). In 2013–2014, Connecticut, Maine, and Vermont are the only three states that have their own laws governing the labelling of GM and processed foods. The

cultivation and commercialization of GM crops and foods are tightly regulated by three federal agencies, namely, the United States Department of Agriculture (USDA), Environmental Protection Agency (EPA), and Food and Drug Administration (FDA) (Bernauer and Meins 2003). The USDA is involved in environment and plant protection from various agents such as agricultural pests, weeds, and diseases under the Plant Protection Act. Commercialization of a new GM plant is required to obtain prior approval from the USDA through its auxiliary body: Animal and Plant Health Inspection Service (APHIS). Applicants are required to follow three standard procedures, namely, notification procedure, permit application, and nonregulated status application (Wolt et al. 2016). In 1994, Flavr Savr® tomato was the first GM crop approved for commercial cultivation. APHIS has so far approved 17,000 applications for environmental release trials of agricultural GMO crops and has granted nonregulated status for 96 GM plants (e.g., corn, tomato, etc.) (Fernandez-Cornejo et al. 2014). The parent organization involved in the development of a particular GM plant or product needs to furnish vital information's related to the donor organism, the recipient organism, and the individual GM ingredients; expression pattern of the introduced genetic material and the molecular machineries was used to develop the product.

The *EPA* works in accordance with the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). It partakes in the supervision of production, marketing, and sales of pesticides and biopesticides (EPA 2015). The EPA regulates the entry of all GM plants that are pesticidal in nature. The FIFRA defines "plant-incorporated protectant (PIP)" as any substance used for pest control and produced indigenously within the plant. These also include selectable markers, genetic materials (foreign gene), and their specific expressed proteins used for biopesticide production. For example, commercialization of a GM Bt crop, the Bt protein, as well as the respective *Bt* gene which encodes the Bt protein needs to be assessed by the EPA. NewLeaf potato is the first PIP-incorporated plant to be granted permission for commercial cultivation by the EPA in the year 1995 (Reed 2014).

The third regulatory agency is the FDA which conducts safety assessments of the GM crops in relation to human and animal consumption. They regulate veterinary drugs and both GMO and non-GMO food products other than meat, poultry, and processed egg products which fall under the jurisdiction of the USDA under the Federal Food, Drug, and Cosmetic Act (FFDCA) (FDA 2015a). GMO food labelling is also regulated by the FDA; however, it holds a voluntary approach where it is not mandatory to reveal the GM ingredients (FDA 2015b).

The FDA granted approval for Golden Rice cultivation in May 2018 with the aim to help Bangladesh and the Philippines overcome vitamin A malnutrition (Lynas 2018). Similarly, Huahui1, developed by a Chinese team in Huazhong University, Hubei Province, has successfully obtained the safety clearance certificate in January 2018. Huhui1 is resistant to rice stem borer, and its nutritional components are not compromised in the transgenic variety (Reuters 2018).

7.4 Regulation in Europe

Food safety assessments and regulations of GM foods is a top agenda for the European Union and its member states (Urnov et al. 2018). The entry of any new GM crop is stringently regulated by the directives laid down by the European Food Safety Authority (EFSA). The EFSA is assisted in its functioning by the competent regulatory authorities of independent member states. The scientific recommendations pertaining to each application is forwarded and approved by the European Commission and EU member states (EFSA 2014) (Table 4), headquarters in Brussels. Risk assessments of the GMOs with regard to environmental release, heath of humans and animals, and consumer and farmer's perspective are evaluated extensively by the EFSA before recommending the commercialization of any GM product. The strength of the scientific evaluation panel of EFSA is 21 independent experts who review all GM product requisitions.

Legislation on GMOs was adopted to protect the rights of people and environment under the following articles: Articles 168 (Public Health), 169 (Consumer Protection), and 191 (Environment) as depicted in the Treaty on the Functioning of the European Union (TFEU) (EFSA 2010). The two important directives that govern the assessment of all GMOs are Regulation No. 1829/2003 (GM Food and Feed) and Directive 2001/18/EC (Deliberate Release into Environment of GMOs). Moreover, legislation on GM crops and products would promote its circulation and availability within the EU and its member states. Each member state holds the privilege to constitute its own law and also regulate the import and export of any specific GM crops in its territory even though that particular GM crop is approved at the EU level. In EU legislation, export of agricultural GM crops, food, and processed products fall under Regulation (EC) No. 1946/2003 (Transboundary Movements of GMOs) (Halford 2019; Lucht 2015).

The EFSA and the regulatory authorities of member states coordinate through a comprehensive, transparent network of 250 scientific experts from 100 organizations during assessment of GMOs. Each applicant is required to furnish detailed information about the inserted gene, method of transformation, expression pattern in plants, human or animal consumption trials, and environmental release (in case of GM foods and feed). In October 2018, the EFSA passed an ordinance that directed all applicants to incorporate details if Sanger sequencing or next-generation sequencing (NGS) technique is employed in the development of GMOs.

The EU has sanctioned field trial approval for 2404 GM plant varieties from 1992 till date. In April, 2015 since 1992, the EU has approved commercial cultivation of ten new GM cultivars of the following plant species: maize (MON 87460), cotton (T304-40; LLCotton25xGHB614), soybean (MON 87705, MON 87708, MON 87769, MON 30542, BPS-CV127-9), canola (MON 88302), and sugar beet (MON 88913) (EC 2015). The EU also has laws that govern the labelling of GM crops, foods, and products derived from GMOs. GMO labelling is prioritized by the EU member states as it reflects the human rights under European Constitution as well as

Table 4 List of regulatory authorities under EU Commission and its member states for assessment of GMOs

S. No.	Member state	Regulatory organizations
1	Austria	Federal Ministry of Health; Umweltbundesamt
2	Belgium	Flanders Institute for Biotechnology
3	Bulgaria	Agrobioinstitute—Bulgaria RAC FCH, MAF
4	Croatia	University of Zagreb
5	Czech Republic	Ministry of the Environment of the Czech Republic
6	Denmark	Ministry of Environment—Environmental Protection Agency
7	Estonia	Ministry of the Environment, Estonia; Tartu University
8	Finland	Ministry of Social Affairs and Health
9	France	High Council for Biotechnology (HCB)
10	Germany	German Federal Agency for Nature Conservation (BfN)
11	Greece	Ministry of Rural Development and Food, DG of Agriculture
12	Hungary	Ministry of Agriculture
13	Ireland	Environmental Protection Agency (EPA)
14	Italy	National Institute for Insurance against Accidents at Work (INAIL)
15	Latvia	Ministry of Agriculture—Food and Veterinary Service (FVS)—Scientific Expert Committee on GMO RA
16	Lithuania	Ministry of Environment of the Republic of Lithuania—Department for Protection of Nature
17	Luxembourg	Division de la securite alimentaire—Direction de la Sante
18	Malta	Malta Environment and Planning Authority (MEPA)—Genetically Modified Organisms (GMOs) and Biosafety
19	Netherlands	Institute for Public Health and the Environment (RIVM)
20	Norway	Norwegian Scientific Committee for Food Safety
21	Poland	Warsaw University of Life Sciences—Faculty of Horticulture and Landscape Architecture
22	Portugal	Faculty of Pharmacy, University of Portugal
23	Slovak Republic	Food Safety Authority—Ministry of Agriculture and Rural Development
24	Slovenia	Ministry of the Environment and Spatial Planning
25	Spain	Ministerio de Economía y Competitividad—AgenciaEstatalConsejo Superior de InvestigacionesCientíficas (CSIC)—Centro de InvestigacionesBiologicas (C IB)
26	Sweden	National Board of Agriculture
27	UK	Food Standards Agency (FSA)

right to knowledge. Moreover, it is a deliberate attempt from the regulatory authorities to create public awareness to improve the consumer perspective of GM crops and their biosafety (EC 2001; Delwaide et al. 2015).

8 Factors Affecting Commercialization of GM Foods

The advent of recombinant DNA technology and genetic engineering contributed significantly toward the development of GM crops. In present, GM crop cultivation is active in 26 countries and the total area under expanded from 1.7 million hectares (1996) to 185.1 million hectares (2016) in a span of 20 years, distributed in 26 countries and regions (James 2016). The acceptance and commercialization of GM crops and foods are dependent on four cardinal factors: (1) acceptance by farmers, (2) consumer acceptance, (3) political barrier (Huesing et al. 2016). The abovementioned factors vary considerably in different countries and are influenced by its regulatory policies, individual people, and their cultural background (Rodrguez-Entrena and Salazar-Ordonez 2013). For example, survey suggests GM foods are more readily accepted in the USA, Brazil, India, and China in comparison to the EU (Hudson et al. 2015).

8.1 Acceptance by Farmers

Recent surveys on GM crops demonstrate that farmers in developed as well as developing countries are benefiting immensely from adopting GM crop production. For successful integration of any GM crop into the market, farmer acceptance is paramount as they are the ones who will grow these crops (Schreiner and Lataczc-Lohmann 2015). Thus, its commercialization is significantly influenced by farm scale and location, farmer knowledge, financial status of farmer, gender, soil topology, government policies and support (in terms of incentives and degree of regulation), available seeds, and equipment (Guehlstorf 2008). There are several reports on the positive impact GM crop cultivation had on the economic and social welfare of farmers and their families. Such commercially cultivated crops include herbicide-resistant (HR) soybean, Golden Rice, Bt maize, Bt cotton, Bt-HR maize, and Bt-HR cotton (Raney 2006; Lucht 2015). Meta-analyses results and periodic reviews of GM crop cultivation highlighted reduction in yield loss due to pest attack and pesticide application, increase in gross, and net income and positively impacted the health of the farmers (due to decreased exposure to toxic pesticides) (Mannion and Morse 2013; Klumper and Qaim 2014; Racovita et al. 2015; Todua and Gogitidze 2017).

Klumper and Qaim (2014) evaluated 147 cultivars from three HR GM plant species (soybean, maize, and cotton) and insecticide-resistant (IR) (*Bt* maize and *Bt* cotton) plant species grown across 19 countries. They reported that farmers' income was increased by 69%. This huge profit margin was attributed to increase in yield output (21.5%) and decrease in pesticide cost (38%). In some cases, the production cost of GM crops exceeded the cost incurred for non-GM crops; however, the gross income profit margin was significantly high in case of GM varieties due to increased yield (Areal et al. 2013).

It is evident that adoption of GM crop cultivation can boost the financial status and health of farmers which in turn leads to their social welfare. However, lot of effort has to be directed toward making GM seeds economically feasible for

small-scale farmers low on capital investment (Azadi et al. 2016). Though GM crop cultivation reduces various input costs, Finger et al. (2011) reported alarming differences in the cost of transgenic *Bt* cotton seeds in South Africa (97%), the USA (222%), and India (223%) in comparison to its non-GM variety.

8.2 Consumer Acceptance

The destination of GM crops after it exists in the farm can range from the market place for human consumption or animal consumption as feed. Commercialization of GM crops is directly proportional to the level of consumer acceptance (Aerni et al. 2011; Deodhar et al. 2007). Various factors have been identified that govern the attitude of consumers toward adoption of GM foods. These factors include consumer's willingness to pay (WTP), product knowledge, individual belief, type of product, environmental impact, heath issues, product pricing, consumer risk perception, and finally trust (Lefebvre et al. 2019; Cui and Shoemaker 2018; Arvanitoyannis and Krystallis 2005; Huffman 2003).

There are several statistical models designed to evaluate consumers' perspective of GM crops based on the above parameters. These models include benefit risk analysis (BRA), theory of planned behavior (TPB), attitude model, and attitude change model (Mather et al. 2012; Zhang et al. 2010; Wilson et al. 2004). In general, all statistical analysis on consumer acceptance of commercially available GM crops and foods depicted low consumers' WTP for GM foods in comparison to foods derived from non-GM crops. Surveys on US and European customers showed that European consumers were more hesitant in adopting GM foods. This skeptic attitude has been linked to misconceptions, negative campaigning, limited awareness, lack of knowledge about GMOs, and government initiative.

Mandatory labelling of food products derived from GMOs is quintessential for consumer acceptance. In the USA, only 25% of the populations are aware of consuming GM foods, but in reality, close to 90% of them have eaten GM foods unknowingly (Lucht 2015). This ignorance is due to the lack of mandatory labelling of GM foods in the USA and can potentially hinder the growth of GM food crop market. In such scenario, a lot is expected from the local government and regulatory authorities in terms of generating proper public awareness leading to public acceptance of GM foods.

8.3 Political Barrier

The political parties and their affiliations are pivotal for commercial cultivation of GM crops and their acceptance in the society. In majority of the countries cultivating GM crops, political sects routinely intervene with the functioning of the regulatory authorities, and reports of destruction of crops, agricultural equipment, government property, and farmland are common (Rommens 2010; Dahabieh et al. 2018; Arujanan and Teng 2018). Every country can be grouped into three categories

(adopters, conflicted, and opposed) based on their political intervenes in acceptance of GM crops.

The adopter countries (e.g., Spain, Denmark, the Netherlands, Sweden, the UK, etc.) actively promote cultivation of GM crops, and they invest heavily in GM technologies and train their farmers in breeding these GM crops. The countries that are classified under "conflicted" include those which maintain dual standards on adoption of GM crops. In such countries (France, India, Germany, etc.) despite active support from farmers, scientists, and regulatory authorities, there is considerable resistance from political parties and nongovernmental organizations (NGOs). The last category consists of countries (Austria, Greece, Hungary, etc.) where policymakers, stakeholders, and government reject the idea of GM technology primarily due to conservative ideologies.

In country like India, dual governance (national and state level) is an area of major concern when it comes to constituting GM crop policies (Ramaswami 2007). The state governments can constitute their own law and can approve or ban cultivation of a particular GM crop though it is approved in some other State. For instance, in June 2013, the national regulatory body (GEAC) approved field trials for 45 GM rice varieties to the developing company (Bayer Bioscience Limited) across India, but the company is still awaiting permission for the same at the state level (Sethi 2013; Kurmanath 2013).

9 Conclusion

Conventional breeding alone cannot cater to the growing food demands of the global population. The situation is further challenged due to the frequent exposure to abiotic stress, changing climatic pattern, less rainfall, and loss of farmable land. Crops developed by genetic engineering are being considered worldwide to increase crop production, and extensive research is being conducted to develop GM crop varieties with superior traits. So far, majority of research has been limited to the development of herbicide- or pest-resistant GM plants. Using GE, various other traits like nutrient enhancement and allergy resistance can also be targeted. In this regard, development of Golden Rice has been a landmark achievement in the field of agricultural biotechnology as it can potentially eliminate vitamin A malnutrition in developing and underdeveloped countries. Similarly, other nutrients or mineral fortification (e.g., iron fortification in rice) can be achieved in the near future. However, successful commercialization of GM crop in any country is heavily dependent on the GM crop policy, stakeholders, government initiative, consumer's perspective, and farmer acceptance. The role of the incumbent regulatory authorities is paramount in developing positive public awareness among farmers as well as consumers. Moreover, the political and economic landscape of the countries also determines the success of GM crop cultivation.

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