S. M. Paul Khurana
Rajarshi Kumar Gaur *Editors*

Plant Biotechnology: Progress in Genomic Era

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Foreword

To address the advances of plant biotechnology, the editors, Prof. S. M. Paul Khurana and Prof. R.K. Gaur, have undertaken the thorny assignment of capturing the status and future trends in the various fields of agriculture and food and nonfood plant production systems. *Plant Biotechnology: Progress in Genomic Era*, delivered by the proponents of agricultural biotechnology, offers a wealth of information about the scientific breakthroughs and discoveries aiming to meet the global challenges of the diminishing amount of arable land as well as energy shortage, malnutrition, and famine. Many eminent and erudite scholars such as Prof. Klaus Ammann, Prof. Ajit Verma, Prof. S. K. Khare, Prof. A. N. Pathak, Dr. Swarup Chakrabarty, Dr. G. P. Singh, Prof. T. Satyanarayana, Prof. Poonam Singh (Nigam), Dr. Kishor Gaikwad, Dr. Senjuti Sinharoy, and Prof. Yuri Dorokov have addressed various aspects of plant biotechnology and provided important contributions for the book. The book consists of four parts, i.e., (i) *Gene and Genome*, (ii) *Biofuel and Bioremediation*, (iii) *Plant as Medicine and GE for (Plant) Stress*, and (iv) *Disease and Crop Management*.

Plant biotechnology comprises a distinct science of deriving valuable products from cells, tissues, and entire plants. The field also involves the exploitation of plants and bioprocess applications in different fields of human activities such as energy production, environmental protection, and industrial use of natural resources. Development of successful biotechnology applications requires thinking across boundaries. The interdisciplinary nature of the field becomes evident in the book, as it covers both traditional and recent developments in the fields of microbiology, mycology, and plant pathology. Plant biotechnology innovations will work in practice only if they can be combined with established strategies and common agricultural practices. If successfully completed, moved through the maze of regulatory safety processes and accepted by consumers, they will continue to shape the future of global agriculture and sustainability of agricultural production.

Various powerful genome sequencing and editing technologies have initiated a new era in plant molecular breeding. It has become increasingly possible to understand the connections between phenotypes and genotypes and to describe gene and protein functions underlying the desired traits. The first part excellently familiarizes the reader with the basic concepts and current technologies of plant genetic engineering. It paves the way for the coming parts dedicated to the introduction of advanced plant biotechnology applications aiming to develop sustainable bioenergy

production, biodegradation, plant-derived health products, and plant disease management. Each of these areas is essential for the short- and long-term success of plant biotechnology and for solving global problems. To exemplify, novel sources of biofuels are urgently required to avoid the use of fossil fuels, the main reason for global increases in atmospheric CO_2 concentrations and climate change.

The book consists of 27 enlightening chapters, which contain numerous beautiful and revealing illustrations helpful for the reader to grasp the essence of the message. Throughout the book, the approaches have been scrutinized with a critical eye as is characteristic for dedicated science professionals. I am confident that this excellent book provides an insightful overview of the prospects and challenges of plant biotechnology both to researchers and to students in this fascinating field. I hope that many readers of the book will become informed advocates of plant biotechnology.

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Kristiina Mäkinen

Preface

The present growth of biotechnology in the current research often overshadows the emerging role of plant biotechnology, due to (having) dramatic developments in the last decade. This would certainly overcome and complement the earlier standard procedures. The book *Plant Biotechnology: Progress in Genomic Era* consists of 27 chapters, divided into 4 major parts of modern biotechnology:

(i) Gene and Genome
(ii) Biofuel and Bioremediation
(iii) Plant as Medicine and GE for (Plant) stress
(iv) Disease and Crop Management

All parts share a search into the regulation of genes responsible for specific qualitative or quantitative traits. As with any new revolutionary science, biotechnology needs to be continuously monitored and regulated for the benefit of the humanity. Each chapter has been written by distinguished scientists, having made significant contributions and pioneers/leaders in the field. All the articles present the opinion of the authors and their viewpoints about the powerful tools of the advancement of biotechnology.

Part I, Gene and Genome, provides the background information on the status of studies in this field and on the recent methodological developments in plant genetic engineering. The questions regarding regulatory issues of GM crops, especially those concerning the novel technologies used in plant molecular breeding, are discussed in Chap. 1. With the advent of various genome sequencing technologies, it is now possible to understand the connections between the phenotypes and genotypes and to describe the gene and protein functions in the desired traits. To save time, money, and effort, the subsets of genomes can be prepared for targeted sequencing by enriching the genome area of interest (Chap. 2). Chapter 3 reveals how recent developments in sequencing strategies have made it possible to obtain the full sequence of the complex hexaploid genome of wheat, which is globally the second most important cereal, for its improvement. The accumulating sequence data will advance the possibilities to improve crops also by modeling biological phenomena and studying them at the system(s) level as envisioned in Chap. 4. The contributors of Chap. 5 have also expressed concern whether the full genome

approaches in the development of next-generation crops can be exploited or not due to the strict regulation against GM crops.

For studying different genomes, several editing tools, such as ZFNs, TALENs, and CRISPR systems, and bioinformatics are used to discuss their revolutionary applications in precision molecular breeding and functional genomics research (Chaps. 6, 7, and 8).

In Part II, Biofuel and Bioremediation, many novel approaches of microbial bioremediation, including bioelectric technology, and biosurfactants are discussed. This part deals with the plant oils that can be used for the production of biodiesel and their physical and chemical properties (Chap. 9). Subsequently, various processes of fermenting cellulosic hydrolysates, using microbial strains, are explained along with the properties of biodiesel obtained (Chap. 12). Also, the different wastes that are used for producing bioelectricity (Chap. 11) and actinomycetes for soil remediation (Chap. 13) and the use of nanosystems as a carrier and delivery system of various essential oils on the target pathogen are discussed (Chap. 10).

In Part III, Plant as Medicine and GE for (Plant) stress, Chap. 14 summarizes current research dealing with medicinal properties and health benefits of *Withania somnifera* with a focus on antioxidant, anticancer, and antimicrobial properties, while Chap. 20 describes defense mechanism and diverse actions of fungal biocontrol agents against plant biotic stresses. Besides, clinical trials and action mechanism of potent compounds extracted from lichens are also described (Chap. 15). It is apparent that nanotechnology offers a wide range of applications and is a highly promising technology for revolutionizing (modern) agriculture (Chap. 17). Chapter 16 describes the aloe vera plantlets under controlled experimental conditions in order to analyze its potential on morphogenesis and secondary metabolites from the test plant. The contributors of Chap. 18 introduce model of legume species that have been used to expand our understanding of the traits associated with root nodule symbiosis (RNS) in plants. Chapter 19 presents an overview of recent advances on the development and application of CRISPR/Cas9 system in plants.

In Part IV, Disease and Crop Management, Chap. 21 summarizes future research on ROS through classical as well as advanced biotechnological methods for a better understanding of plant biology. Chapter 24 describes at length on methods and the prospects of P-TMAs which are especially important for individualized cancer therapy, as well as cases of bioterrorism and pandemics. Furthermore, Chap. 26 provides a comprehensive account on the various diagnostic techniques available for citrus greening/HLB and also discusses the recent advancements in its detection. Various plant viruses have also been described as used for gene silencing vectors and the next-generation vectors (Chap. 22) and RNA gene silencing which focus on the perspectives for utilizing this mechanism as a tool for control of viruses in plants (Chap. 23). Chapter 25 discusses ddPCR applications of plant pathogens using citrus pathogens in duplex and triplex assays. Precise information on chloroplast-virus interaction as developed for disease control strategies and genetically engineered plants with better photosynthetic efficiency and yields is given in Chap. 27.

We wish to express our deep gratitude to all the contributing authors, including many eminent scientists worldwide, who are pioneers in plant biotechnology. While

every effort was made to avoid ambiguity and to maintain uniformity and/or consistency in style, the presented results, ideas, and organizational details of the chapters still reflect personal opinion and preference of the respective authors.

We are highly grateful to the many reviewers, colleagues, and friends, involved in the venture, for their help, advice, and cooperation as well as to the Springer Nature for their kind assistance and ungrudging patience.

Finally, with love and affection, we are also deeply indebted to our families for their patience and understanding.

Gurgaon, India — S. M. Paul Khurana
Gorakhpur, India — Rajarshi Kumar Gaur

Contents

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Editors

Prof. (Dr.) S. M. Paul Khurana had his PhD in 1969 on Papaya Viruses and his 2-year postdoctoral research (1970–1972) on Advanced Plant Virology at Kyushu University, Fukuoka, Japan, with Prof. Zyun Hidaka as JSPS Fellow. He also availed GOI DBT Overseas Fellowship for 1 year at the University of Minnesota, St Paul (USA), with Prof. EE Banttari and specialized in Immunodiagnostics (March 1987–April 1988). Moreover, he worked at the Central Potato Research Institute (CPRI), Shimla, since 1973, as Scientist/Sr. Scientist; Principal Scientist and Head, Virus/Seed Pathology (1976–1982/1988), Principal Scientist and Head, Plant Pathology Division (1988–1993); Project Coordinator, AICRP-Potato (1994–2004); Director CPRI, Shimla (2002–2004); and Vice Chancellor, Rani Durgavati University, Jabalpur (2004–2009). He also served as Visiting Consultant for CIP/FAO (1992, 1996, 1997) and then as the Director of Amity Institute of Biotechnology, AUUP, Noida (2009–2010), and moved to Amity University Haryana at Gurgaon, where he served as Dean, Science, Engineering, and Technology (2013–2016), & Professor of Biotechnology since 2016 to present, in August 2010 for establishing the Institute of Biotechnology (2010–2015). In addition, he is an internationally recognized Plant Virologist/Pathologist and Biotechnologist having 53 years of experience, 230 research papers, and 100+ reviews, guided 16 PhDs, edited 18 books, etc.

Dr. Rajarshi Kumar Gaur earned his PhD in 2005, now Professor, Department of Biotechnology, Deen Dayal Upadhyaya Gorakhur University, Gorakhpur, Uttar Pradesh, India. His PhD was on molecular characterization of sugarcane viruses, viz., mosaic, streak mosaic, and yellow luteovirus. He received MASHAV Fellowship in 2004 from the Israel Government for his postdoctoral studies and joined The Volcani Centre, Israel, and BenGurion University, Negev, Israel. In 2007, he received the Visiting Scientist Fellowship from Swedish Institute Fellowship, Sweden, for 1 year to work in the Umeå University, Umeå, Sweden. He has made significant contributions on sugarcane viruses and published 130 national/international papers and presented nearly about 50 papers in the national and international conferences. He was awarded as Fellow of Linnean Society, London. Currently, he is handling many national and international grants and international collaborative projects on plant viruses and disease management.

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Part I

Gene and Genome

Selected Innovative Solutions for the Regulation of GM Crops in Times of Gene Editing

1

Klaus Ammann

Abstract

The analysis of the structure of the regulatory discourse needs a look behind the curtain about reasons for the dissent, and we need to acknowledge the 'Genomic Misconception': Transfer of transgenes similar to natural mutation. The conclusion is to have a critical look at the present-day regulation and shift to a professional discursive structure of the regulatory rules. The unfortunate decision of the European Court to include all Gene Editing products hinders progress but encourages also to aim at a basic restructuring of the present-day regulation laws instead of only minor corrections. One of the important discursive elements is to include cultural responsibilities of modern agriculture in its broadest aspects.

Keywords

GM crops · Gene editing · Regulatory discourse · Genomic misconception

1.1 Introduction

If we want to escape years-long fruitless debates on biotechnology and biodiversity, we have to do more than just to deplore the debate full of artificial (or imagined) contrasts, the main arguments are summarized below. The debate needs a professional *discursive structure* and we must embrace different kinds of knowledge, and new solutions should not be excluded, on the contrary: in new regulatory structures surprising new discoveries of better crops and in the science of GM safety also have to be anticipated. Basically, a mutual understanding of the different views on

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agricultural strategies (from organic farming to the application of new breeds may stimulate the debate and lead to innovative solutions.

In their publication of Zetterberg and Edvardsson Björnberg (2017), the authors come up with a programmatic summary, which can well serve here as a motivation to go ahead with a courageous proposal for a regulatory change of GM crops:

> *In recent years, the EU legislation on genetically modified (GM) crops has come under severe criticism. Among the arguments are that the present legislation is inconsistent, disproportionate, obsolete from a scientific point of view, and vague in terms of its scope. In this paper, the EU GM legislation (mainly the "Release Directive", 2001/18/EC) is analyzed based on five proposed criteria: legal certainty, non-discrimination, proportionality, scientific adaptability, and inclusion of non-safety considerations. It is argued that the European regulatory framework does not at present satisfy the criteria of legal certainty, non-discrimination, and scientific adaptability. Two ways of reforming the present legislation toward greater accommodation of the values expressed through the proposed criteria are briefly introduced and discussed.* From Zetterberg and Edvardsson Björnberg (2017)

With the necessary courage and organized workforce those plans can be realized in a few months of intensive work, and it should also be possible to push the solutions through in the complex system of European and international regulation.

1.2 The Structure of a Regulatory Discourse

1.2.1 First: Look Behind the Curtain

We need to see behind the curtain and focus on the main driver elements behind the debate. The industry, together with important farmer organizations, wants to see better results of the new breeds in the field for commercial marketing. The scientists focus on facts, strive for innovation and progress in agricultural breeding, and they believe in new solutions to fight the hunger in the world still existing Council for Agricultural Science and Technology (CAST) et al. (2017). Decisive opposition comes from professional NGOs like Greenpeace and Friends of the Earth, most often with arguments which are not supported by science. Both opponent sides build on heavy financial support and are reluctant to lose their expensive structural organization. Scientists often do not understand that a discourse on modern breeding including the public institutions is an absolute necessity. NGOs also fear that public support will faint, a support which is still of very important dimensions: Bouillon (2014). Recently, part of the GM opposition deplores to lose the debate related to the more precise methods of Gene Editing which might be more acceptable to the public and politics. GM-Opponents still consider the modern breeds full of risks but are unable to present convincing facts Steinbrecher and Paul (2017). The debate is often carried in a merciless way, major players risk major loss of income by losing the debate Miller et al. (2008). The main driver behind opponent campaigns is often *diffuse fear*, built on questionable interpretations of substantial equivalence and sustainability and full of additional false arguments constructing negative but unsubstantiated effects of modern breeding. But such negative contributions counting on the natural fear mongering for the public, are contradicted heavily by breeding

optimism, here one example dealing only with the great genomic potential of wild relatives: Wettberg et al. (2018): The conclusions:

> *Collections of wild relatives of crops will be most useful to breeding programs if they reflect the breadth of adaptations present in natural populations, which we argue is best accomplished when collections span the full geographic and environmental range of the species. Our collection expands both the genomic diversity and environmental range of the two closest wild relatives of chickpea, increasing the size of the collection by over an order of magnitude. The variation in substrate, elevation, and climatic range encompassed by the collection increases the likelihood that the assembled germplasm contains variation in phenology, drought, heat and cold stress. Indeed, we observe phenotypes that are correlated with environmental variation in the form of seed color crypsis and responsiveness to drought, and we have identified variation in seed nutrient density, phenology, resistance to pod borer, heat tolerance, and water deficit response. We are also actively exploring segregating variation in Fusarium wilt and Ascochyta blight resistance, nitrogen fixation and plant architecture, each of which represent traits that are of great interest for chickpea crop improvement. Our collection also highlights the need for conservation of CWRs. Rapid development in southeastern Anatolia is accompanied by the fragmentation and loss of native landscapes. Two of the populations reported here were lost or fragmented in subsequent years (2014, 2015), while other populations are threatened by human activities.* ***These facts underscore the urgency of the need to collect, characterize, and preserve both in situ and ex situ wild relatives of crops as essential components of humankind's agricultural heritage and future*** *1 McCouch and Crop Wild Relative (2013), 36, Tanksley and McCouch (1997) and 37 Maxted et al. (2012). Comments from Wettberg et al. (2018).*

It will be important to abstain from unilateral thoughts and try to integrate various methods and approaches for a healthy and future-minded agriculture: Ammann (2012a), Dollacker (2018), Van Wensem et al. (2017), and Ricroch et al. (2016a).

1.2.2 Second: The 'Genomic Misconception' of Existing GM Regulations

Not surprisingly, molecular science and unbiased views on agricultural history should be able to ease down the contrasts in this debate, here two of many arguments:

(a) The process of gene transfer is identical, whether done in natural mutation or modern biotechnology, a view supported in the past many times by Nobel Prize Winner Werner Arber Arber (2010), summarized with details of the regulatory history in the *Genomic Misconception*, a review published 2014 by Ammann (2014).

According to latest papers of Werner Arber, Genetic Engineering represents a safe approach for innovations improving nutritional contents of major food crops Arber (2017a, b).

(b) It is on the other side clear that the *application* of the huge potential of the new methods including *Gene-Editing*, will have important consequences in the future of agriculture. There is a plethora of new crop trait possibilities which are already tested or need to be tested, whether involving "foreign DNA" or not, since all new traits done with molecular methods *embrace a certain procedural*

novelty. The present-day politics of many scientists aims at the full exclusion of those very precise Oligo-Mutations which end up without "foreign" DNA in the product, should be fully excluded from regulation Breyer et al. (2009b). This sounds convincing, but a closer look at the methods of Gene Editing will lead to more precautionary conclusions, as shown below. Some insight in the present day debate on regulation of GM crops can be read in a selection of publications – it is nearly impossible to distill out of the considerable variety of regulatory thoughts into a clear, simple concept (see Chap. 4): Zetterberg and Edvardsson Björnberg (2017), Eriksson (2018a, b, Eriksson et al. (2018), Davison and Ammann (2017), Eriksson and Ammann (2017), Ricroch et al. (2016b, Tagliabue et al. (2016), and Tagliabue et al. (2017).

1.2.3 Third: Organo-transgenic Thoughts

The consequences from the Genomic Misconception analysis are the following:

It is fact, after Wood et al. Wood and Lenne (2001), that our main world crops (Rice, Wheat and Sorghum) have been chosen by our ancestors because they already lived in *large monodominant stands*, an important precondition of efficient food production. The often-heard argument that huge monocultures are directly and negatively related to modern breeding has no logic or historic background. On the contrary, modern breeding can be key to conceive a more ecological methodology in agriculture Ammann (2007b, 2012b). In consequence, we need proposals to merge organic farming with its good sides in biodiversity management, but unfortunately having a strict focus on anti-biotechnology and hostility towards industrial farming with its uncritical perspective on production alone – a critical view which in the latest years received a lot of correction also in conventional agriculture: Consequently, it is better to think the unthinkable such as *Organo-Transgenics*: Indeed, organic farming and biotech farming could actually go together under well-defined circumstances – across ideological and commercial barriers. Ammann (2008, 2009), Ammann and van Montagu (2009), about cisgenic potatoes see Gheysen and Custers (2017).

But the regulatory conclusions from 2.1 to 2.3 are, despite numerous proposals published, somehow complex.

1.3 Conclusion: Regulatory Proposals: The Idea of a Dynamically Scalable Regulation

The regulatory views should in consequence not be black and white for part or the whole modern and traditional breeding: A *dynamically scalable regulatory modus* should be more realistic and more acceptable to friends and foes, see Wolt et al. (2010, 2015) and Podevin et al. (2012a) and Wolt (2017). The Gene Editing

methods which finally do not contain any foreign DNA should still be regulated in a modest way for a few years, then released swiftly after and almost certain positive outcome to the world agriculture applications. More details about the Dynamically Scalable Regulation can be checked out in the citations above. One illustration from Jeffrey Wolt explains concisely the strategy.

The anticipated scrutiny of the various regulatory methods is well summarized in Wolt et al. (2015), specifically in its Fig. 1.1 below.

The full text of interpretation in Wolt is given here with some editing of the author for the reason of a precise argumentation for his dynamically scalable regulation scheme in Fig. 1.1, including the citations with some added items:

Regulatory discussion of a wide range of new breeding techniques applied to crop development was initiated in 2011 with an EU-convened international workshop that considered the techniques then available for site-directed genome editing Lusser and Davies (2013b), and Lusser et al. (2011, 2012. Based on the categorizations identified by this group, its elaboration by Podevin et al. (2012b) see also Devos et al. (2014)—and accounting for the emergence of new techniques in the interim—a schema for regulatory characterization specific to genome editing techniques can be described (Fig. 1.1). This schema considers the approach to DSB repairs that are achieved by NHEJ (SDN1), homologous recombination (SDN2) or transgene insertion (SDN3) and whether the technique for introduction of the GEEN is transient *(Category 1),* introduces rDNA within the plant genome with subsequent removal *(Category 2)* or entails stable plant genome integration of rDNA *(Category 3).* The OMM approach produces DSB repaired by NHEJ and therefore is analogous to SDN1 in terms of its regulatory characterization to the extent the

Category / Method	Category 1 Transient expression resulting in site-specific DSB and repair	Category 2 Stable genomic introduction of rDNA with intermediate steps to generate transgene-free null segregants	Category 3 Stable genomic integration of recombinant DNA
SDN1* Site-directed random mutation involving NHEJ	Low	• Low for deletions • Case-by-case for addition • Higher as size of insertion increases	N/A
SDN2 site-directed homologous repair involving one or very few nucleotides	Case-by-case	Case-by-case	N/A
SDN3 site-directed transgene insertion	N/A	N/A	High, moderated for well characterized insertion sites

Fig. 1.1 Relationship of site-directed genome approach to the anticipated degree of regulatory scrutiny of the plant phenotype obtained. ∗Current uses of OMM are analogous to SDN1 in terms of regulatory scrutiny. (From Wolt et al. 2015)

changes are viewed as point mutations and not template insertions: Hartung and Schiemann (2014); Lusser and Davies (2013b). It is somehow plausible to exclude Oligo-Mutations from the usual regulatory scheme, as many US and EU authors conclude. But apart from this solution, indeed rather simple and tempting from the regulatory point of view, this exclusion will meet decisive opposition from many GM critiques such as Steinbrecher and Paul (2017), we propose a differentiated solution, by taking up the views of Jeffrey Wolt et al. within the following three categories.

1.3.1 Category 1 of a New Dynamic Regulation

Techniques involve transient introduction of recombinant DNA using in vitro synthesized nucleic acids and DNA delivery methods that *do not integrate* into the host genome Pauwels et al. (2014). These techniques, therefore, resemble transgenic processes but produce phenotypes that are indistinguishable from plants obtained through conventional plant breeding. The techniques would include site-specific point mutations with oligonucleotides (OMM), site-specific random mutations by NHEJ (SDN1) and site-specific mutations with DNA repair via homologous recombination (SDN2). Novel techniques avoiding the use of rDNA through direct introduction of the nuclease or mRNA encoding the nuclease Baltes et al. (2014), Baltes and Voytas (2015), and Martin-Ortigosa et al. (2014) to catalyze similar mutation events would also fall into this category.

1.3.2 Category 2 of a New Dynamic Regulation

Consists of stable introduction of rDNA into the host genome and an intermediate step involving expression of SDN1 or SDN2 to effect DSBs and repairs. Subsequent breeding selection for null segregants results in phenotypes that are indistinguishable from phenotypes obtained through conventional plant breeding. Therefore, evidence will generally be lacking in the product to indicate a transgenic process was involved in the intermediate step. Plant phenotypes developed by SDN1 methods as described in either of the forgoing categories represent simple point mutations and with few exceptions (Canada) regulators do not consider crops developed by mutagenesis in the same context as GM crops. The regulatory opinions regarding plant phenotypes developed by SDN2 methods are not as clear, as the nature and extent of the edits used to effect the desired change in the phenotype obtained by the technique would influence opinions as to whether the phenotype represented a GM product. For instance, deletions are viewed as less consequential than are additions. And in the case of additions, the greater the number of bases added, the greater the level of regulatory concern. Important in this context is the determination as to whether the NHEJ accomplished by the technique is viewed as a template insertion into the genome Lusser and Davies (2013a).

1.3.3 Category 3 of a New Dynamic Regulation

Category 3 finally involves techniques which result in stable integration of rDNA where 'Genome editing with engineered nucleases' (GEEN) is used to specifically target delivery of a transgene or multiple transgenes through insertion by homologous recombination (SDN3). Current examples of this technique involve the site-directed stacking of transgenes D'Halluin and Ruiter (2013). Thus, they simply represent a refined technique to accomplish transgenesis and would be considered no differently than GM products by regulators. The European Food Safety Authority (EFSA) Panel on Genetically Modified Organisms—an expert panel providing independent scientific advice to EFSA on GMOs—has developed the regulatory opinion that existing EFSA guidance documents apply to the SDN3 technique EFSA GMO Panel (2012), see also other important EFSA-publications: EFSA Gmo Panel Working Group on Animal Feeding Trials (2008), EFSA Guidance (2011), EFSA Guidelines and Renn Ortwin (2012), EFSA Independence (2012), EFSA letter and Paoletti Claudine (2015), and EFSA Opinion (2015). But because the technique can specifically target transgene delivery into the genome, it has the potential to minimize potential hazards associated with gene disruption or regulatory elements in the recipient genome.

*Thus, plants developed using SDN1 methods may require less data for risk characterization than more conventional approaches to transgenesis: s*ummary with edits of the author from Wolt et al. (2015)

> *Summary: Genome editing with engineered nucleases (GEEN) represents a highly specific and efficient tool for crop improvement with the potential to rapidly generate useful novel phenotypes/traits. Genome editing techniques initiate specifically targeted double strand breaks facilitating DNA repair pathways that lead to base additions or deletions by non-homologous end joining as well as targeted gene replacements or transgene insertions involving homology-directed repair mechanisms. Many of these techniques and the ancillary processes they employ generate phenotypic variation that is indistinguishable from that obtained through natural means or conventional mutagenesis; and therefore, they do not readily fit current definitions of genetically engineered or genetically modified used within most regulatory regimes.* ***Addressing ambiguities regarding the regulatory status of genome editing techniques is critical to their application for development of economically useful crop traits. Continued regulatory focus on the process used, rather than the nature of the novel phenotype developed, results in confusion on the part of regulators, product developers, and the public alike and creates uncertainty as of the use of genome engineering tools for crop improvement.*** *From Wolt et al.* (2016)

And from the paragraph of the same text of Wolt et al. 2016 "Needs within the regulated community"

> *The need to rapidly innovate to introduce novel traits in crops is heightened by increased world food demand and increasing use of crops as sources of renewable energy (Edgerton 2009). The opportunity for transgenic crop innovation is limited by regulatory hurdles and continued public unease Pew Initiative et al.* (2015) *and Smyth et al.* (2015). *Transgenic technologies continue to elicit considerable public misunderstanding and mistrust despite 19 years of commercial use and over 181.5 million hectares in production globally in 2014 James (*2014). *Largely in response to effective pressure on the part of a broad spectrum of NGO and activist groups Paarlberg* (2014) *and the continuing public pressure it has engendered, the*

regulatory processes for transgenic GE crops (the so-called GMOs) are largely broken in many parts of the world. Implementation of national biosafety laws is encumbered in the developing world Bayer et al. (2010) *and Okeno et al.* (2013) *and long delays in cultivation approvals are reducing the value of innovation in many regulatory domains Smyth and Phillips* (2014)*. New breeding technologies, especially site-directed genome editing, are viable alternatives to transgenic crop production that provide new opportunities for innovation and which in many cases clearly involve a reduced degree of regulatory oversight. Success in advancing GEEN and related technologies for crop improvement will be limited if public views and regulatory response continues to be captured within the overriding theme of GMOs. The continued reliance on process-based definitions as a guide to regulatory oversight—and the adoption of process-focused language in public discourse—detracts from appropriately gauged approaches toward the regulation of genome-edited crops. Thus, the focus on the nature of the novel plant phenotype/trait is lost as the appropriate paradigm for the safety assessment, which encumbers regulatory approvals for crops derived from both established and emerging plant breeding techniques. Lacking a fuller emphasis on this point means that the public may largely misunderstand genome editing and regulators will be faced with pressure to evaluate these products within existing biosafety frameworks. Fortunately, progress is being made by regulators in shaping sensible and pragmatic approaches toward the application of genome editing for crop improvement but at some point to new product-based paradigms for regulation of new breeding technologies must emerge. From Wolt et al*. (2016)

See also the new table from Wolt (2017).

	Category 1	**Category 2**	**Category 3**
SONI	NHEJ or nucleotide replacement with transient introduction of reagent	NHEJ with stable integration of nuclease-encoding rONA and subsequent NS selection	NHEJ with stable integration of nuclease-encoding rONA
SDN2	HDR with transient introduction of reagent	HOR with stable integration of nuclease-encoding rDNA and subsequent NSselection	HOR with stable integration of nuclease-encoding rONA
SDN3	Transient introduction of reagent with site-directed transgene insertion	Site-directed transgene insertion with stable integration of nuclease-encoding rONA and NS selection	Sit e-directed transgene **insertion with stable** integration of nuclease-encoding **rDNA**

See the explanation from the first paper of Wolt et al. (2016), table from Wolt (2017)

Again, in his latest publication Wolt (2017) Jeffrey Wolt insists (as does the author) on a ***Dynamically Scalable Regulatory Modus*** (Box 1.1).

Genome editing with engineered nucleases (GEEN) is increasingly used as a tool for gene discovery and trait development in crops through generation of targeted changes in endogenous genes. The development of the CRISPR- Cas9 system clustered regularly interspaced short palindromic repeats with associated Cas9 protein), in particular, has enabled widespread use of genome editing. Research to date has not comprehensively addressed genome-editing specificity and off-target mismatches that may result in unintended changes within plant genomes or the potential for gene drive initiation.Governance and regulatory considerations for bioengineered crops derived from using GEEN will require greater clarity as to target specificity, the potential for mismatched edits, unanticipated downstream effects of off-target mutations, and assurance that genome reagents do not occur in finished products.

Box 1.1 Genome-Editing Approaches and Categories from Wolt (2017)

In case-by-case assessments of biotechnology-derived plant products, regulators initially seek understanding as to the egree the methodology employed is familiar, as this helps to inform assessment of risks and uncertainties that may be associated with a given trait within the crop of interest. In terms of genome-edited crops, regardless of the reagent system used, an understanding of potential downstream outcomes for the plant product can be ascertained through consideration of the editing approach and the introduction and fate of the reagent. Wolt et al. (2016)

Accomplishing a genome edit uses a site-directed nuclease (SDN) to cause DSBs. Repair of the DSBs occurs by various mechanisms: NHEJ which randomly insert/deletes one to several bases to cause point mutation (SDNl) and homology-directed repair (HDR) involving native or synthetic template insertion (SDN2) or transgene insertion (SDN3). These approaches represent, respectively, insertion of progressively consequential nucleotide base sequences at a targeted DSB. Approaches involving OMM involve simple nucleotide replacements and are analogous to SDN 1 to the degree the edits are viewed as point mutations and not template insertions. Lusser and Davies (2013a)

Additionally, in accomplishing the genome edit, the technique for introduction of the GEEN may be transient in the form of the protein, protein/RNA complex (e.g., Cas9/gRNA ribonucleoprotein complex), mRNA, or DNA that does not integrate into the host genome (category 1); may introduce nuclease-encoding rDNA in the genome that is subsequently removed through NS selection (category 2); or may involve stable genome integration of nuclease-encoding rDNA (category 3). These categories represent a progression of increasingly consequential procedures ranging from transient insertion of short-lived ribonucleoprotein complexes into the cytoplasm of cells to the introduction of nuclease-encoding rDNA into the host genome.

Together, the approach to gene editing used and the category describing introduction and fate of the nuclease roughly reflect the degree of regulatory uncertainty regarding the derived phenotype. The topology of current regulatory views concerning a given outcome of genome editing in terms of these factors are shown below (as adapted from Wolt and colleagues Wolt et al. 2016*). An additional layer of regulatory consideration with respect to category 2 and category 3 will be whether the design of the reagent has the potential to enable a gene drive.*

Since governance and regulatory decision making involves robust standards of evidence extending from the laboratory to the post-commercial marketplace, developers of genome-edited crops must anticipate significant engagement and investment to address questions of regulators and civil society*. From Wolt* (2017)

1.3.4 Summary: Clearly, There Is Still an Ongoing Regulatory Dispute Among Scientists

Most scientists and an important number of European scientists insist in a non-regulatory status of Oligo-Mutations not containing "foreign DNA", a position which can be seen with some scientific merits: It sounds somehow logic that mutational breeding not containing foreign DNA should not fall under the present day tedious and cumbersome regulation.

But still, the argument cannot be dismissed that after all, the new Oligo-mutation breeds are made with an *over-all new methodology*, which needs a minimum of regulatory proofing. Indeed, research has not addressed comprehensively the potential for mismatched edits and the results are contradictory: Considerable amount of mismatched edits: Fu et al. (2013), Sander and Joung (2014), Schaefer et al. (2017), and Zischewski et al. (2017) low incidence of such edits: Veres et al. (2014) unanticipated downstream effects of off-target mutations, and assurance that genome reagents do not occur in finished products. We should agree therefore to the *Dynamically Scalable Regulatory Modus* as described above. Unfortunately, the European Court has recently taken regulatory decisions which are clearly outside proper scientific thought, details below in Chap. 6.

1.4 Outlook

Recent papers demonstrate with more detailed conclusions and comments about the Regulation of GM crops within Europe, that the scene of new ideas is volatile: Ammann Klaus (2017), Davison John and Ammann Klaus (2017), Eriksson and Ammann (2017), Ricroch et al. (2016b), Tagliabue et al. (2016, 2017), and Tagliabue and Ammann (2018). More interesting ideas and concepts on modern regulation have been published mainly by German and US authors: Hokanson et al. (2018), Raybould et al. (2012), and Roberts et al. (2014). And the question is, why the European Union needs a national GMO opt-in mechanism: Eriksson et al. (2018). In Europe, it is still the question, whether regulatory hurdles for genome editing should be process- or product-based approaches in different regulatory contexts: Sprink et al. (2016), although most authors lean towards a product-view. In the eyes of the author this question depends on the perspective of the treated crop regulation. The divide among scientists often focuses to the regulation of Oligo-Mutations which lack in the final product "foreign" DNA, many follow the US decisions of not regulating those breeds, some, like the author, would like to see instead of such a rather theoretical molecular regulatory divide a *dynamically scalable regulation* as defined above, which includes also the Oligo-Mutations without foreign DNA, but with a minimum stretch of only 2–3 years of regulatory scrutiny – as described above. A very detailed and important debate about the regulation of Gene Editing is given by Jeffrey Wolt 2017: Wolt (2017), see above and remarks below:

Deciding on regulatory needs for 'Genome editing with engineered nucleases' (GEEN) it is not done with the simplistic distinction between products with or

without foreign DNA, the questions on safety situation are more complex, the author agrees fully with Jeffrey Wolt, here his more detailed comments from 2017 (p.220ff).

One concern are the off-target effects, which are still not studied enough: Jacobs et al. (2015), Jeffrey Wolt's comments Wolt (2017).

> *For example, increased editing efficiency is observed over time for genome modifications targeted using CRISPR- Cas9 in cultured soybean embryos. This may indicate continued expression of Cas9. during embryo and plant development and the potential for dose-dependent (concentration x time) effects that may increase off-target mutations. Addressing this will require further consideration off genome analysis of CRISPR-edited plant lines as well as improvements in computational tools, reagent design, and experimental methodology; collectively, these can lead to further insurances regarding the limited potential for unanticipated effects at likely mismatch sites within the genome. From Wolt* (2017)

The other, lesser concerns are the possibilities of Gene Drive events, Wolt's own comments:

> *The creation of gene drives in crop plants or livestock is of lesser concern than applications where gene drives are expressed in wild organisms under open release conditions – as may be the case if gene drives are used to eliminate invasive plant species or overcome pesticide resistance in weedy species. For domesticated plants and animals, there is limited opportunity for uncontrolled gene drive escape and dissemination because of long generation times and control of breeding lines which, respectively, reduce gene drive efficiency and provide a means to observe and remove undesired phenotypes which may be inadvertently developed. Addition,1lly, since domesticated crops and food animals are not competitive with sexually incompatible wild species, the probability is low for environmental establishment of gene drive-bearing crops or livestock. See the important citation: National Academies of Sciences* (2016)*, from Wolt* (2017)

In this complex situation of a multifaceted dispute, it is important to conduct future discourses under the auspices of a *modern discourse strategy*, as already promoted by Churchman (1979, 1984) and Rittel and Webber (1973, 2005).

> *"The search for scientific bases for confronting problems of social policy is bound to fail, because of the nature of these problems. They are "wicked" problems, whereas science has developed to deal with "tame" problems. Policy problems cannot be definitively described.* ***Moreover, in a pluralistic society there is nothing like the undisputable public good; there is no objective definition of equity; policies that respond to social problems cannot be meaningfully correct or false; and it makes no sense to talk about "optimal solutions" to social problems unless severe qualifications are imposed first.*** From Rittel and Webber (1973, 2005)

1.5 The Unfortunate Decision of the European Curia

The decision surprised many:

Hopes were real that the European Court would take a decision along those lines of analysis, as one of the closest experts like *Advocate General Michal Bobek* still in January 2018: (cited in the GAIN Report) GAIN Report et al. (2018)

On January 18, 2018, an Advocate General [Bubak] of the Court of Justice of the EU (ECJ) is expected to publish his legal opinion clarifying how certain innovative plant breeding techniques should be regulated under EU law (Case C-528/16 Confederation Paysanne and Others). This advisory opinion will set out to answer whether and to what extent organisms developed through certain classical and innovative plant breeding techniques, in particular directed mutagenesis, are to be regulated either through the same framework as conventional plant breeding or as genetically modified organisms (GMOs). Presently, organisms developed through classical mutagenesis breeding techniques are regulated as conventional and are exempt from the EU's main GMO regulation, Directive 2001/18/EC. [i]

This ECJ interpretation of the law will have major implications for the future of seed breeding and innovation in Europe, as well as impacts on European agricultural production and global trade. In the ECJ, an Advocate General's legal opinion is non-binding and advisory for the panel of judges who decide the case; but is nonetheless viewed as important in shaping the final determination. The ECJ's Grand Chamber of Judges is expected to issue their findings in summer of 2018. From GAIN Report et al. (2018)

The non-binding opinions of Advocate General Michal Bobek were published on January 18: Michal Bobek Advocate General CURIA (2018a, b).

Abstract and conclusions from January 18 by Michal Bobek.

According to Advocate General Bobek, organisms obtained by mutagenesis are, in principle, exempted from the obligations in the Genetically Modified Organisms Directive Member States are free to adopt measures regulating such organisms provided they respect overarching principles of EU law The 'GMO Directive'1 regulates the deliberate release into the environment of genetically modified organisms (GMOs) and their placing on the market within the EU. In particular, the organisms covered by that Directive must be authorised after an environmental risk assessment. They are also subject to traceability, labelling and monitoring obligations. The Directive does not, however, apply to organisms obtained through certain techniques of genetic modification, such as mutagenesis ('the mutagenesis exemption'). Unlike transgenesis, mutagenesis does not, in principle, entail the insertion of foreign DNA into a living organism. It does, however, involve an alteration of the genome of a living species. The mutagenesis techniques have made it possible to develop seed varieties with elements resistant to a selective herbicide

V. Conclusions

168.In the light of the foregoing considerations, I recommend that the Court answer the questions referred to it by the Conseil d'État (Council of State, France) as follows:

(1) *Provided that they meet the substantive criteria of Article 2(2) of Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC, organisms obtained by mutagenesis are genetically modified organisms within the meaning of that directive; The exemption laid down in Article 3(1) of Directive 2001/18, read in conjunction with its Annex I B covers all organisms obtained by any technique of mutagenesis, irrespective of their use at the date of the adoption of that directive, on the condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms other than those produced by one or more of the methods listed in Annex I B.*

(2) *Council Directive 2002/53/EC of 13 June 2002 on the common catalogue of varieties of agricultural plant species is to be interpreted as exempting varieties obtained by muta-*

genesis from the specific obligations laid down therein for the inclusion of genetically modified varieties in the common catalogue of agricultural plant species.

(3) *Directive 2001/18 does not preclude Member States from adopting measures governing mutagenesis provided that, in so doing, they respect the overarching obligations arising from EU law.*

(4) *Consideration of the fourth question referred has not disclosed any factor of such a kind as to affect the validity of Articles 2 and 3 of Directive 2001/18 and its Annexes I A and I B.*

(All details can be read in the full text of Advocate General Michal Bobek: Michal and Advocate General CURIA 2018b)

But Advocate General Michal Bobek's expectations from January 2018 were nearly fully neglected: Many scientists, breeders, and agri-food industry stakeholders had anticipated with him that the Court would categorize organisms derived from these newer mutagenic techniques as GMOs, but exempt them from the regulatory obligations in the Directive, as also their closest scientific Bubak expert (see above) anticipated:

Unfortunately, the European Court has decided – despite of having heard and read (but obviously not understood) many scientifically sound arguments from Bobek, that Gene Editing should be included without exception and differentiation into the existing EU regulatory law: On July 25, 2018, the Court of Justice of the European Union issued its judgment that organisms created through many newer genome editing techniques are to be regulated as genetically modified organisms (GMOs) in the EU. This decision subjects such organisms, and food and feed products containing these organisms, to an expensive and lengthy approval processes as well as traceability, labelling, and monitoring obligations. In addition to affecting global agricultural trade, this judgment has significant negative consequences for the EU breeding innovation and innovation in agricultural approaches.

Indeed, as a big surprise to many, the Court (Curia Europaea) InfoCuria (2018b) issued in Luxembourg a judgment for the Case C-528/16 taking a very restrictive view of how the EU's main GMO legislation from 2001, EU-Directive (2001) applies to organisms created by new plant breeding techniques such as CRISPR/Cas or Talen.

The full preliminary text published from the Curia website in English, (see citation above with the link for the French version, which is actually the legally binding original text).

The main four Curia statements:

1. *Do organisms obtained by mutagenesis constitute genetically modified organisms within the meaning of Article 2 of Directive [2001/18/EC] of 12 March 2001,*[1] *although they are exempt under Article 3 of and Annex IB to the directive from the obligations laid down for release and placing on the market of genetically modified organisms? In particular, may mutagenesis techniques, in*

[1] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC — Commission Declaration (OJ 2001 L 106, p. 1)

particular new directed mutagenesis techniques implementing genetic engineering processes, be regarded as techniques listed in Annex IA, to which Article 2 refers? Consequently, must Articles 2 and 3 of and Annexes IA and IB to Directive 2001/18 of 12 March 2001 be interpreted as meaning that they exempt from precautionary, impact assessment and traceability measures all organisms and seeds obtained by mutagenesis, or only organisms obtained by conventional random mutagenesis methods by ionising radiation or exposure to mutagenic chemical agents existing before those measures were adopted?

2. *Do varieties obtained by mutagenesis constitute genetically modified varieties within the meaning of Article 4 of Directive 2002/53/EC of 13 June 2002 on the common catalogue of varieties of agricultural plant species,*[2] *which would not be exempt from the obligations laid down in that directive? Or, on the contrary, is the scope of that directive the same as that which under Articles 2 and 3 of and Annex IB to the Directive of 12 March 2001, and does it also exempt varieties obtained by mutagenesis from the obligations laid down for the inclusion of genetically modified varieties in the common catalogue of agricultural plant species by the Directive of 13 June 2002?*
3. *Do Articles 2 and 3 of and Annex IB to Directive 2001/18/EC of 12 March 2001 on the deliberate release into the environment of genetically modified organisms constitute, insofar as they exclude mutagenesis from the scope of the obligations laid down in the directive, a full harmonization measure prohibiting Member States from subjecting organisms obtained by mutagenesis to all or some of the obligations laid down in the directive or to any other obligation, or do the Member States, when transposing those provisions, have a discretion to define the regime to be applied to organisms obtained by mutagenesis?*
4. *May the validity of Articles 2 and 3 of and Annexes IA and IB to Directive 2001/18/EC of 12 March 2001 with regard to the precautionary principle guaranteed by Article 191(2) of the Treaty on the Functioning of the European Union, in that those provisions do not subject genetically modified organisms obtained by mutagenesis to precautionary, impact assessment and traceability measures, be called in question, taking account of the development of genetic engineering processes, the appearance of new plant varieties obtained by means of those techniques and the current scientific uncertainty as to their impacts and the potential risks they represent for the environment and human and animal health?*
English text from InfoCuria (2018b)

The full text of the decision does not offer more relevant details: InfoCuria (2018a).

It is interesting to read in the full text that the opinion of Bobek is indeed properly cited, but there is no real argument given for its full rejection and the report is ignoring the rich argumentation of Bobek, except for the following remarks:

[2] *Council Directive 2002/53/EC of 13 June 2002 on the common catalogue of varieties of agricultural plant species (OJ 2002 L 193, p. 1)*

64 As the Advocate General has noted in point 161 of his Opinion, it would be inconsistent to impose obligations, with regard to the environmental risk assessment, on genetically modified varieties within the meaning of Directive 2002/53 from which they are explicitly exempted by Directive 2001/18.

When you go to the original opinion paragraph 161 of the full paper of Bobek, such interpretation as above should be further-on discussed:

161. First, there should be internal consistency within Directive 2002/53. Thus, Article 4(4) must be read in conjunction with Article 7(4)(a) of the same directive. As notably submitted by the French Government, it would be inconsistent to impose the same kind of obligations, with regard to the environmental risk assessment, on genetically modified varieties under Directive 2002/53 whereas they are explicitly exempted there-from under the GMO Directive. Therefore, the mutagenesis exemption should also apply in the context of Directive 2002/53.

The two following remark No. 162 and 163 of Bobek (2018) should also be noted:

162. Second, there should also be external consistency between Directive 2002/53 and secondary law instruments regulating GMOs. Unlike the GMO Directive and other secondary law instruments which do expressly exclude mutagenesis from their scope (54: See Article 2(5) of Regulation No 1829/2003 and Article 3(2) of Regulation No 1946/2003), Directive 2002/53 does not primarily concern GMOs. It regulates varieties of agricultural plant species generally. It certainly does not aim at governing GMOs in the first place, but merely touches upon them incidentally, in order to emphazise their specificity and the fact that they are regulated by specific rules that shall prevail over general ones.

163. It follows that Directive 2002/53 cannot be seen as lex specialis with regard to the GMO Directive. It is rather the other way around. Directive 2002/53 is the lex generalis applying to a whole set of varieties of agricultural plant species, including genetically modified varieties. It would hardly be conceivable that products exempted from the obligations laid down in the specialized, GMO-specific legislation would have to comply with equivalent substantive obligations on the basis of a piece of EU legislation that primarily legislates in a different field and only incidentally touches upon GMOs.

Many scientists, breeders, and agri-food industry stakeholders (including the author) had wrongly anticipated that the Court would categorize organisms derived from these newer mutagenic techniques as GMO's, but exempt them from the strict, expensive and time-consuming regulatory obligations in the existing Directive.

Hope is still alive, that the EU commission will overturn or heavily revise this judgement, which is built on a restricted set of arguments of law and that the commission comes to a more tenable and scientifically solid solution.

Indeed, after a first shock we can view the court's decision more rational and hope that a detailed analysis will also lead the way out of the debacle. The search for new regulatory solutions should be influenced by the early critique of regulatory decisions taken within the Cartagena Protocol. Chassy et al. (2004), De Greef (2004), ENB-IISD-Cartagena-Negotiations (1999), Gupta and Falkner (2006), McHugen (2006), Miller Henry (2017), Morris (2008), Nobs et al. (2003), and Pythoud (2004).

In an early paper from 2009 Breyer et al. already claim that Oligo-Mutations (OMMs) not containing foreign DNA should be excluded from GM regulation: Breyer et al. (2009a)

1. *OMM must be considered as 'leading to genetic modifi cation' in the meaning of the EU Directives.*
2. *All reviews clearly indicate that the process is a type of gene repair and not homologous recombination.*
3. *The technique does not involve the introduction or integration of new genetic material in organisms, but alters chromosomal or episomal sequences in situ in their natural genetic background.*
4. *OMM should therefore not be considered as a recombinant nucleic acid technique in the meaning of the EU Directives.*
5. *We are also of the opinion that the nucleic acid molecules used in the technique (oligonucleotides) should not be considered recombinant nucleic acid molecules. OMM does not make use of any vector system.*
6. *Delivery of the oligonucleotide in the cell can involve micro-injection or micro-encapsulation (in liposomes), although other techniques such as electroporation or particle bombardment are more commonly used.*
7. *OMM can be considered as a form of mutagenesis, a technique that is excluded from the scope of the EU regulation. From Breyer et al.* (2009a)

The overwhelmingly negative reactions about the European court's decision were loud and clear, here only a small selection of letters, publications and official statements, most of them criticizing heavily the Court's decision, including some strong statements to keep certain CRISPR methods out of regulation even before the Curia decision was taken: Tim and Raines (2017), BVL (2017), Callaway (2016), Hackenbroch and Schmiedel (2017), House of Commons (2017), Miller(2017), Scheufele and David (2017), ScienceMediaCentre (2018), transGEN and Fladung (2018), White Samuel (2017), and Whitechurch Odile (2017).

Here we present only the first eight expert opinions on the EU court's decision published in the ScienceMediaCentre (2018).

The European Court of Justice has ruled that organisms obtained by mutagenesis – a set of techniques which make it possible to alter the genome of a living species without the insertion of foreign DNA – are GMOs and are, in principle, subject to the obligations laid down by the GMO directive.

Prof ***Cathie Martin, Group Leader, John Innes Centre, said:*** *"It is important to point out the wider implications of this ruling (wider than simply its impact on traits engineered using New Breeding Technologies). The important point is that this ruling ignores assessment of the safety of the trait developed, and rules only on the technology used. So introduction of higher yielding crops engineered by mutagenesis (traditional or by NBT) could be blocked by NGOs in the absence of an approved environmental impact evaluation!*

"This is going to impact plant breeding in Europe hugely and negatively."

Prof Wendy Harwood, Crop Transformation Group, Department of Crop Genetics, John Innes Centre, said:

"Every single plant on our planet is here because of mutations occurring during evolution. Human society as we know it, relies on the deliberate selection of mutations to improve food crops.

"The European Court of Justice opinion that organisms obtained by mutagenesis are GMOs and therefore subject to the obligations of the GMO directive is a disappointing setback for the use of valuable new technologies in crop improvement.

"Older mutagenesis techniques that have a long safety record are exempt from this obligation. The same outcomes can be achieved using newer, faster and more precise mutagenesis methods as using the older techniques. Treating the plants derived in different ways is not a logical approach based on the scientific evidence. This decision could have major negative

impacts on our ability to respond rapidly to the challenges of providing sufficient, nutritious food under increasingly challenging conditions."

Dr Nicola Patron, Head of Synthetic Biology, Earlham Institute, said:

"The European Court of Justice has ruled that certain new techniques and methods used to induce precise mutations which alter the genetic material of organisms are GMOs and therefore subject to the obligations of the GMO directive. This is not an approach based on scientific evidence. Mutagenesis is a natural phenomenon responsible for the genetic diversity that can been seen in all living organisms. Humans have used different technologies to induce mutations in plants to increase genetic diversity and improve the agronomic qualities of crops for almost a century; the same outcomes can now be achieved using faster, more efficient and precise mutagenesis methods. In most cases, it will not be possible to determine which technique was used to induce the mutation. This decision may negatively impact our ability to respond to the challenge of securing sufficient food for our growing population in a changing climate. It may also hinder the competitiveness of the EU's biotechnology sector."

Prof Nick Talbot, Deputy Vice Chancellor, and Professor of Molecular Genetics, University of Exeter, said:

"This ruling by the CJEU is a mis-guided and retrograde step that is not based on any scientific evidence. Mutation occurs all the time in all organisms. Many modern crop varieties were generated over the last several decades by random mutagenesis in which there was no control on secondary effects. Precise modern gene editing technologies allow accurate, predictable changes to be made in a genome. To classify gene edited crops as GMOs and equivalent to transgenic crops is completely incorrect by any scientific definition. By adopting the precautionary principle in such a mis-guided and short-sighted way, Europe is again being denied the opportunity to innovate and lead in the development of beneficial, environmentally-friendly agriculture for the next century."

Prof Denis Murphy, Professor of Biotechnology, University of South Wales, said:

"This ruling has potentially important implications for the regulation of the exciting new technique of genome editing both in the EU and elsewhere. Essentially the ruling, which is derived from a case involving plants, would appear to cause all new genome edited organisms to be regulated as if they were derived from classical 'GM' or transgenic methods as developed in the 1980s.

"This will potentially impose highly onerous burdens on the use of genome editing both in agriculture and even in medicine, where the method has recently shown great promise for improving human health and well-being.

"It is of course important that, like any new biotechnology, genome editing is properly assessed and regulated according to evidence-based scientific criteria. However, by simply lumping together genome editing with the completely different GM/transgenic biotechnologies, the CJEU has missed a historic opportunity to create a new regulatory framework for this new biotechnology.

"In the rest of the world genome editing will continue to be used for human welfare, whether in curing hitherto intractable genetic diseases or in helping developing countries grow better crops. But sadly much of Europe might miss out on such opportunities if genome editing becomes effectively impossible to use in the EU."

Prof Sophien Kamoun, Senior Group Leader and Professor, The Sainsbury Laboratory, said:

"This ruling ignores advances in plant bioediting that make this technology more precise than so-called 'conventional mutagenesis'. Bioediting can be also be used to recapitulate natural variations into cultivated varieties of crops."

"This ruling closes the door to many beneficial genetic modifications such as breeding of disease resistant plants that require much less pesticide input. "A sad day for European plant science."

Penny Maplestone, CEO of the British Society of Plant Breeders, which represents the agricultural plant breeding industry in the UK (a list of BSPB members is at http://www.bspb.co.uk/members.php), said:
"The Court has ruled that all plants derived from any type of mutagenesis are GMOs according to the legal definition in the Directive. It rules further that the exemption that the Directive gives to exclude mutants from the need to be regulated as GMOs applies only to well established types of mutagenesis and not to the latest plant breeding techniques of extremely precise targeted mutagenesis, even where these result in plants that could have been produced by traditional plant breeding methods and contain no foreign DNA. The outcome means that all plants derived by genome editing will be caught by the GMO regulations.
"This ignores the opinion of the Advocate General and is very bad news for plant breeding innovation in Europe. It seems likely that the potential of these innovative methods to deliver crop varieties with better disease resistance, enhanced nutrition, higher yields and resilience to extreme weather events such as the drought we are currently experiencing, will be lost to farmers and consumers in Europe. Other parts of the world have already given a green light to plant breeding innovation through exciting scientific developments like CRISPR Cas9 and are forging ahead. It is a deep disappointment to see Europe closing the door to plant breeding innovation at a time when we need it as we never have before to address the challenges of food production, climate change and environmental protection."
Prof Jonathan Jones, Plant Scientist, The Sainsbury Laboratory, said:
"Commenting as a scientist not a lawyer, and relying on the text of the press release rather than the ruling, this outcome looks unhelpful for Europe, food security and international trade. Notably, it is framed around the idea that because these methods can be used to confer herbicide resistance, any application of these methods should be considered a GMO. This excludes a host of benign and beneficial applications for disease resistance and stress tolerance that are without conceivable harmful side effects. Other jurisdictions, notably US and China, are moving rapidly to facilitate exploiting new editing methods for crop improvement, and one can anticipate that as a consequence of this ruling, investment in these technologies will depart the EU for more supportive countries. From ScienceMediaCentre (20180725)

These citations come from some of the world's leading British scientists in this research field, they leave no doubt about the sturdy, clearly scientific rejection of the Curia-Decision.

Excellent comments and concrete proposals come also from Purnhagen et al. (2018) in Nature Biotechnology:

After heavily criticizing the Curia decision, the group of leading scientists makes some important proposals:

Option 1*. Member states take action and regulate the use of whole plants and viable materials from plants created with mutagenic NPBTs for release into the environment, such as for field trials and cultivation, under their own laws, which affect only their jurisdiction. In this case, member states have a wide array of regulatory measures at their disposal, ranging from information obligations to authorization requirements. Within these options, member state practice may also vary. In case of an authorization requirement, some member states may grant approval immediately, whereas others may ask for additional information, restrict cultivation or ban cultivation Such a framework has been set by Germany in response to a company's submission of a herbicide-tolerant oilseed rape line developed with ODM to the national competent authority for approval 12 BVL*

(20170228). ***Companies may prioritize which jurisdictions they apply in and which they do not. In the case of foods, food ingredients originating from NPBTs will be subject to the EU food law framework, in particular the rules of the General Food Law. Member states' discretion to regulate foods derived from mutagenic NPBTs is narrower in this area, as most provisions of EU food law follow the rules of maximum harmonization*** *13 Purnhagen and Rott* (2014).

Option 2. *EU institutions take action and introduce legislation to regulate mutagenic NPBTs at the EU-wide level. For example, following a proposal of the European Commission, the EU Parliament and the EU Council would vote on adapting the directive's exemption list to exclude specific mutagenic NPBTs. As a consequence – mutagenic NPBTs would be regulated in a manner comparable to regular GMOs, which require authorization under the directive. Alternatively, the EU could decide to develop parallel EU-wide legislation for NPBTs specifically, with its own requirements and EU-centralized procedures.*

Option 3*. EU institutions frame member state laws for mutagenic NPBTs. Again, following a proposal of the Commission, the EU Parliament and the EU Council vote on the application of a general legislative framework to govern NPBTs at the EU level, but leave it to the member states to decide how to specifically apply them. In this case EU law could regulate general principles that govern member states' regulatory decisions also for environmental release following, for example, the regulatory technique used in the EU's General Food Law.*

Option 4*. No action will be taken and mutagenic NPBTs will remain exempt and therefore unregulated in terms of specific GMO law. Nevertheless, companies have to comply with general national regulations, such as the national seed and environmental laws similar to laws covering non-GM crops, as well as those from the General Food Law, including particularly those for 'novel foods'. We believe option 4 is unlikely to be taken up EU-wide. Member states most probably will call for specific regulations reflecting their societal and economic needs, as has been seen in parallel cases relating to the cultivation of GM crops in national territories 14 Beckmann et al.* (2006).

Options 2 and 3 *are also unlikely to deliver a well-working framework—past experience has shown that consensus among European policymakers is difficult to find on any type of new legislation concerning bioengineered products 15 Smart et al.* (2015). ***Option 1*** *may thus be the preferred choice to serve the interests of the public and consumers without stifling innovations in the crop-breeding sector. Within this framework, member states can choose to implement measures, which will likely result in a competitive patchwork of regulations. Some member state regulations will be more demanding than those in other member states. Depending on a host of societal and economic factors 16 Eriksson et al.* (2018)*, the national authorities may dial up or dial down the stringency of their oversight accordingly. Ultimately, as all the different member state frameworks come into line with one another, a more efficient regulatory environment may come into being, resulting in de facto harmonization 17 Kerber and Van den Bergh* (2008). *In summary, we conclude that the AG's opinion creates the opportunity to move away from the current regulation of new crop varieties toward a more scientific, risk-based and decentralized strategy, as outlined above. If the opinion is followed, the possibility opens up of a more transparent case-by-case approach at the member state level. This will not only assure the safety of newly introduced NPBT varieties for consumers and the environment, but also respect differing public opinions and farm business structures across the EU. From Purnhagen et al.* (2018)

1.6 Conclusions, Possible Solutions

The follow-up of many comments is summarized by Tagliabue and Ammann Tagliabue and Ammann (2018), the abstract if giving the major thoughts:

For the coming years it is suggested by Tagliabue and Ammann to follow along two work-avenues of regulatory development along the targets of a radical regulatory reform:

> *A radical reform of the agri-food biotech regulation in the EU is considered in many quarters (mostly by academia and industry) as a pressing necessity. Indeed, two important decisions (by the European Court of Justice and by the Commission) on the legal status of the so-called New Breeding Techniques are expected shortly. In order to clarify some basic aspects of the complex scenario, after a brief introduction regarding the "GMO" fallacy, we offer our point of view on the following facets:*
>
> (1) *A faulty approach is frequent in the discussion of the agri-food regulation;*
> (2) *NBTs, genome editing may lead to the disappearance of the "GMO" meme;*
> (3) *Beyond health and safety issues: socio-economic considerations;*
> (4) *Sustainability: the comprehensive, meaningful starting point of a positive reform;*
> (5) *The theoretical and legal basis for the reform are already contained in the EU's general guidelines to legislation.* From Tagliabue and Ammann (2018).

The author is not fully decided on whether Oligo-Mutations lacking in their final stage foreign DNA should be excluded from regulation, however, he rather leans towards a short-time inclusion for regulation, since after all, this is still a new, basically untested product, see the detailed debate under Chap. 5.

The proposals of a regulatory follow-up of the author:

A: Quick mending of the most important legal text hindering a reasonable progress in modern agricultural breeding. If you read the full text of the new InfoCuria decision, you will discover that there is considerable space of interpretation and good reasoning for swift and small regulatory changes, but also it offers ample reasoning for a full revision of European (and worldwide) regulation of modern breeding.

B: Full revision of the GM crop regulation of Europe, this means a complete overhaul of the existing paragraphs, and also make some decisions to exempt selected GM crops already existing which are used without any problems since decades (this may be done already under A).

It should be adapted to international developments and come to a more pragmatic solution under full respect of safety issues. This will take more time, but not years as some suggest, a new European working group with an efficient timetable and some finances for the practicalities of the work (not for writing fees) will solve the problems less than 1 year to come.

Within both amendment pathways, it is of crucial importance that some text adaptations will allow also for future, unexpected breeding developments. As history of Science has always told us, *we must reserve ample room for unexpected*

surprises in scientific progress. It is also important to build bridges between traditional and modern agriculture on all levels. It is also of great importance, to decide on exclusion of certain well known and worldwide spread transgenic crops from regulation, due to the ca. 20-years of positive experience of some traits spread on millions of hectares and cultivated over decades.

1.7 Science as a Cultural Responsibility

It is high time to come from the usual factual dispute wars to a cultural perception of science on a higher level. We too often reduce the debate on modern Science on factual arguments, and we forget in a tragic way that true Science can only be founded in a broad and deep perception of culture.

I comprehensive summary of the present-day difficult situation comes from Philipp Aerni (2018). His introduction to the Chap. 3 of the book edited by James HR. Jr. "Ethical Tensions from New Technology, the Case of Agricultural Biotechnology" comes in a deep-going analysis right to the point of all the falsehood of the present-say GMO debate, especially in Europe.

Introduction

Concerns about the risks of genetically modified organisms (GMOs) in agriculture are often framed as an ethical rather than a scientific issue. The ethical issue revolves around the question 'who are the winners and losers?' In today's debate the answer appears to be obvious: the winners are profit-seeking global companies such as Monsanto, while the losers are believed to be consumers, local farming communities and the environment that are exposed to an untested technology. Yet, after more than 20 years of experience with genetic engineering in commercial agriculture, the technology is hardly untested. In fact, new gene-editing techniques may become the next-generation breeding technologies that render the term 'GMO' obsolete.

In view of rapid technological change and industrial transformation, the public debate on GMOs, shaped by the discursive power of the opponents, seems to be increasingly anachronistic. This chapter addresses the shift of discursive power from supporters to opponents of GMOs over the past three decades from a social psychology as well as a political economy perspective. In this context, the view that opponents of GMOs are driven by ethical concerns is challenged. Opportunistic behavior by professional anti-GMO factions will be illustrated through two specific political debates on GMOs in the Swiss and the EU Parliaments. The two cases reveal how the public narrative against the case of GMOs allows opponents to conceal their private agendas behind a 'common weal rhetoric', which portrays them as selfless representatives of the common or public interest. However, as I will show, the credibility of this common weal rhetoric stands and falls with the credibility of the term 'GMO', which has developed a life of its own, very much detached from the technology itself. From Philipp (2018).

Another one of the profound texts analyzing the situation about cultural elements in the Science debate comes from Harris (2001):

In his own words:

*"**Summary:** Cultural Materialism, published in 1979, was Marvin Harris's first full-length explication of the theory with which his work has been associated. While Harris has developed and modified some of his ideas over the past two decades, generations of professors have looked to this volume as the essential starting point for explaining the science of culture to students. Now available again after a hiatus, this edition of Cultural Materialism contains the complete text of the original book plus a new introduction by Orna and Allen Johnson that updates his ideas and examines the impact that the book and theory have had on anthropological theorizing."*

***Within the text**: Cultural materialism is a non-Hegelian strategy whose epistemological assumptions are rooted in the philosophical traditions of David Hume and the British empiricists – assumptions that led to Darwin, Spencer, Tylor, Morgan, Frazer, Boas, and the birth of anthropology as an academic discipline. Yet cultural explained instead of vice versa as has hitherto been the case" (see p. 141). The "materialism" in "cultural materialism" is therefore intended as an acknowledgement of the debt owed to Marx's formulation of the determining influence of production and other material processes. Now I am aware that a strategy which calls itself materialism runs a special risk of being dismissed by the general public as well as by the academic professorate. Materialism is a dirty word among the young, who aspire to be idealistic in their thought and behavior. Materialism is what happens to you when you abandon your ideals and sell out. (Never mind that the more money people make, the more likely they are to think of themselves as idealists.) But cultural materialists have idealistic motives just like everyone else. And as for pure, unselfish devotion to humankind, rightly or wrongly, a large segment of world opinion today ranks Marx as the equal or superior of Jesus Christ. Needless to say, the technical distinction between cultural materialism and idealism has nothing to do with such invidious comparisons. It refers exclusively to the problem of how one proposes to account for sociocultural differences and similarities. Despite the negative images the word "materialism" evokes, I would be intellectually dishonest not to use it. From Harris Marvin* (2001)

And from another side, but the same view of an enlarged cultural dimension of the debate on GM crops comes from Bruno Latour, who until lately, was a strong defender that scientific facts should be seen as a mere product of scientific enquiry, and that facts are networked, they stood or fell not on the strength of their inherent veracity but on the strength of the institutions and practices that produced them and made them intelligible. If this network broke down, the facts would go with them. But in a notable interview with the New York Times, he made a major shift in his view of Science: Kofman Ava and Latour Bruno (2018)

In the summer of 1996, during an international anthropology conference in southeastern Brazil, Bruno Latour, France's most famous and misunderstood philosopher, was approached by an anxious-looking developmental psychologist. The psychologist had a delicate question, and for this reason he requested that Latour meet him in a secluded spot — beside a lake at the Swiss-style resort where they were staying. Removing from his pocket a piece of paper on which he'd scribbled some notes, the psychologist hesitated before asking, "Do you believe in reality?"

"For a moment, Latour thought he was being set up for a joke. His early work, it was true, had done more than that of any other living thinker to unsettle the traditional understanding of how we acquire knowledge of what's real. It had long been taken for granted, for example, that scientific facts and entities, like cells and quarks and prions, existed "out there" in the world before they were discovered by scientists. Latour turned this notion on its head. In a series of controversial books in the 1970s and 1980s, he argued that scientific facts should instead be seen as a product of scientific inquiry. Facts, Latour said, were "networked"; they stood or fell not on the strength of their inherent veracity but on the

strength of the institutions and practices that produced them and made them intelligible. If this network broke down, the facts would go with them.

Still, Latour had never seen himself as doing anything so radical, or absurd, as calling into question the existence of reality. As a founder of the new academic discipline of science and technology studies, or S.T.S., Latour regarded himself and his colleagues as allies of science. ***Of course, he believed in reality, he told the psychologist, convinced that the conversation was in jest. From the look of relief on the man's face, however, Latour realized that the question had been posed in earnest. "I had to switch interpretations fast enough to comprehend both the monster he was seeing me as," he later wrote of the encounter, "and his touching openness of mind in daring to address such a monster privately. It must have taken courage for him to meet with one of these creatures that threatened, in his view, the whole establishment of science****. Cited from Kofman Ava and Latour Bruno* (2018)

It is promising to see the change of the basic Science debate, it is not anymore on facts alone, but involves all the important other fields of science, including social sciences and philosophy. This is where the ardent and seemingly ever-lasting GM crop debate has to adopt a different basic view and attitude. The author of this text has long since advocated this kind of broader debate view: Ammann Klaus (2007a, b, 2013), Miller et al. (2008)

There are also leading GM crop scientists who deal seriously with ethical thoughts of modern agricultural breeding: Ricroch et al. (2018): The decisive sentences: "We have then refocused on moral 'imperatives', such as freedom, justice and truth. Doing so does not resolve all conflicting views, but allows a gain in clarity in the sense that the ethical concerns are shifted from a technology (and its use) to the morality or amorality of various stakeholders of this debate."

It is generally accepted that transgenesis can improve our knowledge of natural processes, but also leads to agricultural, industrial or socio-economical changes which could affect human society at large and which may, consequently, require regulation. It is often stated that developing countries are most likely to benefit from plant biotechnology and are at the same time most likely to be affected by the deployment of such new technologies. Therefore, ethical questions related to such biotechnology probably also need to be addressed. We first illustrate how consequentialist and nonconsequentialist theories of ethics can be applied to the genetically modified organism debate, namely consequentialism, autonomy/consent ethics (i.e. self-determination of people regarding matters that may have an effect on these people) and virtue ethics (i.e. whether an action is in adequacy with ideal traits). ***We show that these approaches lead to highly conflicting views. We have then refocused on moral 'imperatives', such as freedom, justice and truth. Doing so does not resolve all conflicting views, but allows a gain in clarity in the sense that the ethical concerns are shifted from a technology (and its use) to the morality or amorality of various stakeholders of this debate.*** *From Ricroch et al.* (2018)

It all comes together in a revival of ***Enlightement***, here defined and outlined after Pinker (2018).

Let us consider seriously the definition of Enlightement, as given by Steven Pinker, a reminder which seems to be highly necessary in the times of growing materialism:

The Enlightenment principle that we can apply reason and sympathy to enhance human flourishing may seem obvious, trite, old-fashioned. I wrote this book because I have come to realize that it is not. More than ever, the ideals of reason, science, humanism, and progress need a wholehearted defense. We take its gifts for granted: newborns who will live more than eight decades, markets overflowing with food, clean water that appears with a flick of a finger and waste that disappears with another, pills that erase a painful infection, sons who are not sent off to war, daughters who can walk the streets in safety, critics of the powerful who are not jailed or shot, the world's knowledge and culture available in a shirt pocket. But these are human accomplishments, not cosmic birthrights. In the memories of many readers of this book — and in the experience of those in less fortunate parts of the world — war, scarcity, disease, ignorance, and lethal menace are a natural part of existence. We know that countries can slide back into these primitive conditions, and so we ignore the achievements of the Enlightenment at our peril. In the years since I took the young woman's question, I have often been reminded of the need to restate the ideals of the Enlightenment (also called humanism, the open society, and cosmopolitan or classical liberalism). It's not just that questions like hers regularly appear in my inbox. ("Dear Professor Pinker, What advice do you have for someone who has taken ideas in your books and science to heart, and sees himself as a collection of atoms? A machine with a limited scope of intelligence, sprung out of selfish genes, inhabiting spacetime?") It's also that an obliviousness to the scope of human progress can lead to symptoms that are worse than existential angst. It can make people cynical about the Enlightenment-inspired institutions that are securing this progress, such as liberal democracy and organizations of international cooperation, and turn them toward atavistic alternatives. The ideals of the Enlightenment are products of human reason, but they always struggle with other strands of human nature: loyalty to tribe, deference to authority, magical thinking, the blaming of misfortune on evildoers. The second decade of the 21st century has seen the rise of political movements that depict their countries as being pulled into a hellish dystopia by malign factions that can be resisted only by a strong leader who wrenches the country backward to make it "great again." These movements have been abetted by a narrative shared by many of their fiercest opponents, in which the institutions of modernity have failed and every aspect of life is in deepening crisis — the two sides in macabre agreement that wrecking those institutions will make the world a better place. Harder to find is a positive vision that sees the world's problems against a background of progress that it seeks to build upon by solving those problems in their turn. From Pinker Steven (2018)

In Chap. 3, Pinker defends his concept of *enlightement* (probably the biggest achievement of earlier European culture) with arguments, which should be read and re-assumed to-day as utmost important lines:

PART III REASON, SCIENCE, AND HUMANISM The ideas of economists and political philosophers, both when they are right and when they are wrong, are more powerful than is commonly understood. Indeed, the world is ruled by little else.

"Ideas matter. ***Homo sapiens*** *is a species that lives by its wits, concocting and pooling notions of how the world works and how its members can best lead their lives. There can be no better proof of the power of ideas than the ironic influence of the political philosopher who most insisted on the power of vested interests, the man who wrote that "the ruling ideas of each age have ever been the ideas of its ruling class." Karl Marx possessed no wealth and commanded no army, but the ideas he scribbled in the reading room of the British Museum shaped the course of the 20th century and beyond, wrenching the lives of billions. This part of the book wraps up my defense of the ideas of the Enlightenment. Part I outlined those ideas; part II showed they work. Now it's time to defend them against some surprising enemies — not just angry populists and religious fundamentalists, but factions of mainstream intellectual culture. It may sound quixotic to offer a defense of the Enlightenment*

against professors, critics, pundits, and their readers, because if they were asked about these ideals point-blank, few would disavow them. But intellectuals' commitment to those ideals is squirrely. The hearts of many of them lie elsewhere, and few are willing to proffer a positive defense. Enlightenment ideals, thus unchampioned, fade into the background as a bland default, and become a catch basin for every unsolved societal problem (of which there will always be many). Illiberal ideas like authoritarianism, tribalism, and magical thinking easily get the blood pumping, and have no shortage of champions. It's hardly a fair fight. Though I hope Enlightenment ideals will become more deeply entrenched in the public at large — fundamentalists, angry populists, and all — I claim no competence in the dark arts of mass persuasion, popular mobilization, or viral memes. What follow are arguments directed at people who care about arguments. These arguments can matter, because practical men and women and madmen in authority are affected, directly or indirectly, by the world of ideas. They go to university. They read intellectual magazines, if only in dentists' waiting rooms. They watch talking heads on Sunday morning news shows. They are briefed by staff members who subscribe to highbrow papers and watch TED talks. They frequent Internet discussion forums that are enlightened or endarkened by the reading habits of the more literate contributors. I like to think that some good might come to the world if more of the ideas that trickle into these tributaries embodied the Enlightenment ideals of reason, science, and humanism. From Pinker Steven (2018)

Another aspect of the dynamics how valuation is influenced by ***pride*** has been studied by Sznycer et al. (2017)

Significance: *Cross-cultural tests from 16 nations were performed to evaluate the hypothesis that the emotion of pride evolved to guide behavior to elicit valuation and respect from others. Ancestrally, enhanced evaluations would have led to increased assistance and deference from others. To incline choice, the pride system must compute for a potential action an anticipated pride intensity that tracks the magnitude of the approval or respect that the action would generate in the local audience.* ***All tests demonstrated that pride intensities measured in each location closely track the magnitudes of others' positive evaluations. Moreover, different cultures echo each other both in what causes pride and in what elicits positive evaluations, suggesting that the underlying valuation systems are universal.***

Abstract: *Pride occurs in every known culture, appears early in development, is reliably triggered by achievements and formidability, and causes a characteristic display that is recognized everywhere. Here, we evaluate the theory that pride evolved to guide decisions relevant to pursuing actions that enhance valuation and respect for a person in the minds of others. By hypothesis, pride is a neurocomputational program tailored by selection to orchestrate cognition and behavior in the service of: (*i*) motivating the cost-effective pursuit of courses of action that would increase others' valuations and respect of the individual, (*ii*) motivating the advertisement of acts or characteristics whose recognition by others would lead them to enhance their evaluations of the individual, and (*iii*) mobilizing the individual to take advantage of the resulting enhanced social landscape. To modulate how much to invest in actions that might lead to enhanced evaluations by others, the pride system must forecast the magnitude of the evaluations the action would evoke in the audience and calibrate its activation proportionally.* ***We tested this prediction in 16 countries across 4 continents (n = 2,085), for 25 acts and traits. As predicted, the pride intensity for a given act or trait closely tracks the valuations of audiences, local (mean r = +0.82) and foreign (mean r = +0.75). This relationship is specific to pride and does not generalize to other positive emotions that coactivate with pride but lack its audience-recalibrating function.***

See the geographical variation, it demonstrates the importance of the factor "pride" in the whole debates properly (Fig. 1.2):

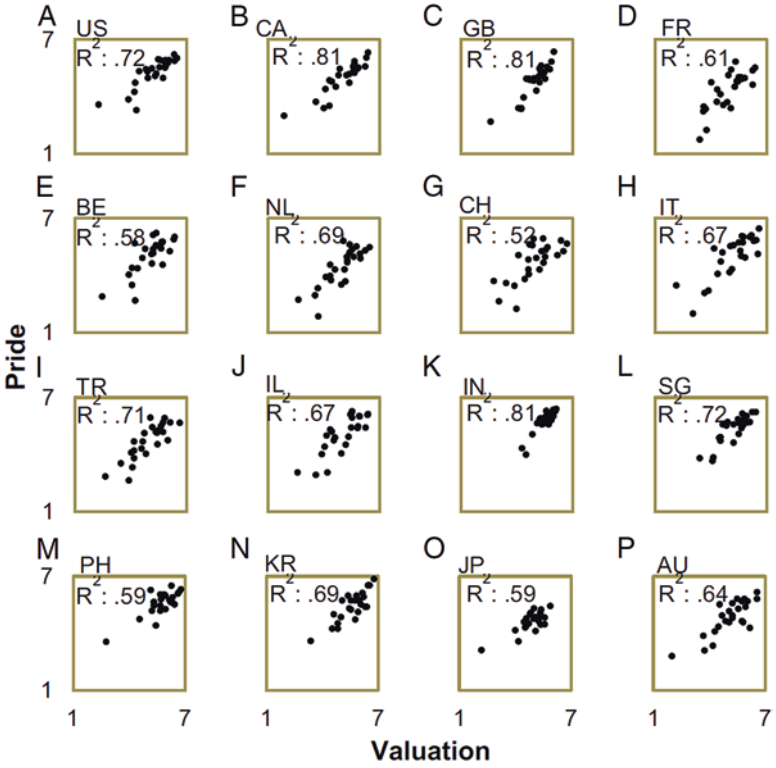

Fig. 1.2 Studies 1a–1p. Scatter plots: pride as a function of valuation. Each point represents the mean valuation rating and mean pride rating of one scenario. Valuation and pride ratings were given by different participants (n = 25 scenarios; effect size: R2 linear). (**a**) United States (US). (**b**) Canada (CA). (**c**) United Kingdom (GB). (**d**) France (FR). (**e**) Belgium (BE). (**f**)The Netherlands (NL). (**g**) Switzerland (CH).(**h**) Italy (IT).(**i**) Turkey (TR). (**j**) Israel (IL). (**k**) India (IN). (**l**) Singapore (SG). (**m**) Philippines (PH). (**n**) South Korea (KR). (**o**) Japan (JP). (**p**) Australia (AU). From Fig. 1.1 in Sznycer et al. (2017)

As a caveat, the one-sided view of agricultural intensification can be seen in a critical way, especially when socio-economic aspects are left out or under-estimated or, as here, not fully included in the statistics: Rasmussen et al. (2018):

> *Land- intensification in agrarian landscapes is seen as a key strategy to simultaneously feed humanity and use ecosystems sustainably, but the conditions that support positive social-ecological outcomes remain poorly documented. We address this knowledge gap by synthesizing research that analyses how agricultural intensification affects both ecosystem services and human well-being in low- and middle-income countries. Overall, we find that agricultural intensification is rarely found to lead to simultaneous positive ecosystem service and well-being outcomes. This is particularly the case when ecosystem services other than food provisioning are taken into consideration. From Rasmussen et al.* (2018)

A final word comes from Biljana Papazov Ammann in (simply forgotten by the author to fill in) here the full additional text: A final word comes from Biljana

Papazov Ammann by introducing a new question culture: Papazov-Ammann (2010) What do we need as visionaries: Progress or Development? This is my question today, as I deal with the topic of Biovisionaries here in the Library of Alexandria. I ask this question because I am convinced that we need to build a new culture of questioning. We need a culture orienting itself by authentic questions. How can we develop taste and the ability to distinguish between those questions which are cognitive, statement- oriented and those which are authentic, close to life and to people? What is more important: cognizance or decision for action? How can we move between Statements and Questions? Statements reflect the need to understand the world. But they are the result of past experience and are often contained in frameworks which are coined by society. They may even protect old routines which hinder innovation. Questions, in contrast to statements, can transform our judgements and prejudices. Questions give birth to energy for new orientation, for a more conscious future. This orientation towards the future, towards vision provokes those choice-questions, and they alone will open the way for an urge to change the world. Visions need people who are free! The quality of freedom is inherent in the question. We must strive for this quality through choice-questions. If we cannot befriend these choice-questions with science, it will disengage from the questioners and will not be human science anymore. Thus we need a new humility of thinking – as it has been wonderfully defined by the German philosopher Heidegger: "The question is the devoutness of thinking." From Papazov-Ammann (2010)

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2 Principles and Implications of Various Genome Enrichment Approaches for Targeted Sequencing of Plant Genomes

Parampreet Kaur and Kishor Gaikwad

Abstract

The higher eukaryotic organisms harbor huge amounts of DNA in their cells that are generally very complex and dynamic in nature. Strategies of sequencing of higher plants have undergone revolutionary changes concurrent with the development of different and newer chemistries. After the advent of NGS technlogies, the approaches to decipher higher plant genomes have evolved in a precise manner to unlock the genetic potential in a targeted manner. Target enrichment refers to the techniques aiming to reduce genome complexity and enrich for specific subset of the genome for sequencing purposes, to deduce a more meaningful and comprehensive data in a fraction of time, cost and effort. Based on the enrichment mode, these approaches and their modifications are classified as PCR-, hybridization-, Restriction enzyme- based and Enrichment for expressed genomic sequences. Since targeted enrichment techniques confers most of the benefits as those of WGS, these are especially useful for target or trait specific studies and are bound to grow for their diverse applications in both reference and non-model crop species.

Keywords

Genome partitioning · Target enrichment approaches · Crop improvement · Sequencing · Plant genomes

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2.1 Introduction

A substantial conservation exists in the overall gene repertoire and relative gene order among angiosperms and gymnosperms, despite the presence of incredible plasticity among their genome sizes. For example, despite 90% of Arabidopsis ESTs sharing homology with loblolly pine, genome size of the former is 162 times less than that of loblolly pine (Kirst et al. 2003). This paradox is referred to C value paradox and defines the absence in correlation of genome sizes with its structural complexity in higher eukaryotes. The variation in the genome sizes and variable gene organization could be reasoned with polyploidization, de-repressed transposition, amplification of non-genic repeat sequences, spontaneous insertions and deletions, gene duplications, genes loss and heterochromatin effect (Hartl 2000).

To understand this variability and its purpose of existence, i.e., translation to the observed phenotype; considerable efforts have been directed towards decoding of whole genomes of organisms through various sequencing techniques. Initiated by Sanger et al. (1977) and Maxam and Gilbert (1977), the sequencing era has seen an exponential growth ever since the emergence of NextGen Sequencers that could be invariably classified into second-, third- and fourth- generation systems and differ in terms of their chemistries, data generation, data acquisition and downstream analysis. The capabilities of these newly developed chemistries such as those of Illumina, Nanopore, PacBio etc. to generate vast amounts of data is far superior to that offered by the first NextGen sequencer, Roche 454 or the traditional Sanger sequencer and has thus dramatically changed the large scale sequencing landscape and is still continuing. Bioinformatics tools have also mirrored the developmental pace of the sequencing chemistries of the NextGen technologies, thereby allowing the acquisition and effective interpretation of the genome sequence data for its incorporation towards crop improvement programs.

The flood of information generated by these sequencing engines has enabled the characterization of complete genomes of various plants such as Arabidopsis, rice, wheat, barley, Medicago, lotus, maize, pigeon pea, soybean etc. either via whole genome shotgun sequencing or BAC by BAC based sequencing approach. For model plants, as well as plants with small genome size, genome characterization at an inter-individual scale (Arabidopsis, Schneeberger et al. 2011; Soybean, Lam et al. 2011; rice, Arai-Kichise et al. 2011), assessing alternative splicing at whole transcriptome level (Arabidopsis, Filichkin et al. 2009) as well as genetic dissection of complex trait variation etc., has been successfully demonstrated. Recently, sequencing of 3000 rice genomes from 89 countries have been successfully completed, allowing one to tap this genomic diversity for future rice crop improvement (The 3000 rice genomes project 2014). Also, whole genome resequencing of 292 accessions of pigeonpea has opened avenues of research to achieve yield and sustainability in this tropical grain legume (Varshney et al. 2017). This new era of NextGen sequencing technologies has made genome sequencing easier, accessible as well as cost effective, thus making genomics an integral part of all branches of life science (Egan et al. 2012).

Though BAC-by-BAC based sequencing is referred to as the gold standard to determine the genome sequence of an organism, the deciphering of complex whole genomes of higher plants such as wheat, sugarcane etc., are not easily amenable. Several strategies were thus deployed to gain access into complex genomes of plants (Bevan et al. 2017) when WGS is either not practical or feasible such as,

(a) Sequencing of diploid progenitors as in strawberry (Shulaev et al. 2011), Brassica (Chalhoub et al. 2014).
(b) Sequencing of sorted and purified chromosome arms as in wheat (The International Wheat Genome Sequencing Consortium 2014).
(c) Reduction of genome complexity as in loblolly pine (Neale et al. 2014).

Techniques employed for the reduction in the genome complexity to aid sequencing of important and/or interesting subset of complex genomes are referred to **as target enrichment or genome partitioning or genomic capture techniques** (Garber 2008) and provides many of the benefits conferred by whole genome sequencing but at a fraction of time, effort and cost. These technologies aim at the reduction of genomic complexity with an objective to isolate specific genomic regions for subsequent multiplexing and NextGen sequencing in a cost- and time- efficient manner as well as simplified data analysis. In contrast to the genome skimming approaches (Straub et al. 2012), based on the assembly of high-copy fraction of total genomic DNA (gDNA) into the nuclear ribosomal cistron (nrDNA), plastome (cpDNA), and individual mitochondrial genes (mtDNA) without genome reduction during library preparation; target enrichment is based on reduction of genome complexity during library preparation. Thus, by virtue of genome skimming approaches one can target high copy number- and repetitive- regions of genome whereas unique fraction of nuclear genomes are targeted for genome enrichment purposes.

This chapter briefly discusses the principles of the various methods employed for target enrichment, their pros and cons, benefits over whole genome sequencing approaches and their utility for crop improvement program.

2.2 Performance Determinants of Target Enrichment Approach

Several methods are available to enrich or partition the genome for target DNA sequences and are evaluated on the basis of several performance metrics (Fig. 2.1). While performing target enrichment, it is very important to determine the ratio of abundance of target region post-enrichment versus pre-enrichment, also referred to as **fold enrichment or enrichment degree** (Mertes et al. 2011). It is also defined as the ratio of coverage of targeted region relative to that of the non-enriched fraction. Among all parameters, **specificity** of target enrichment, referred to as proportion of "target reads" relative to total sequencing reads (related to the degree of enrichment) and **uniformity** i.e., the amount of variation in sequencing depth within and across multiple targets, are critical performance parameters to define the amount of

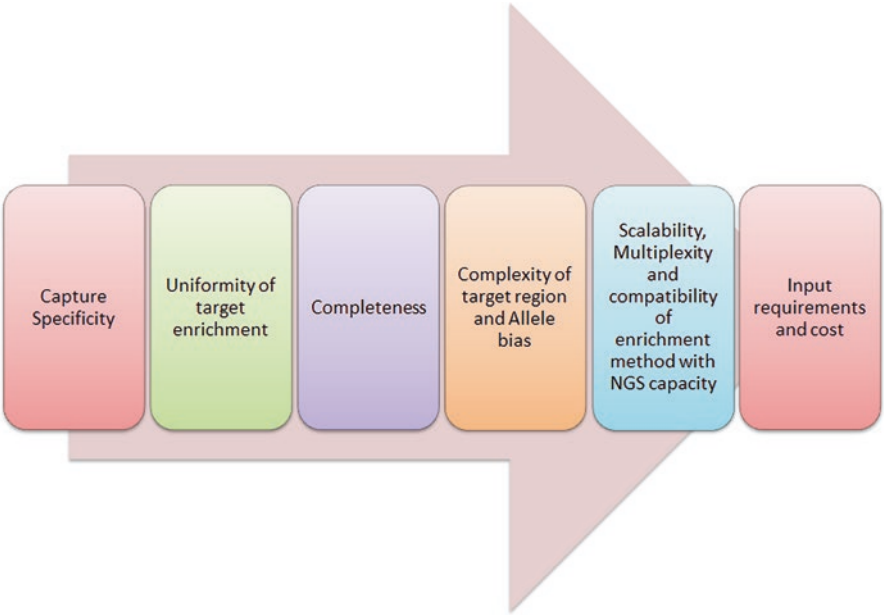

Fig. 2.1 Parameters for consideration of genome partitioning approaches

sequencing required to achieve the required coverage of the target regions. **Completeness** refers to the fraction of the target region measurably captured by a particular strategy. **Allelic bias** refers to preferential capturing of one allele relative to another due to non-uniform capturing efficiency and amplification of two alleles of heterozygous target/variants. Other determinants viz. **Scalability, multiplexing, compatibility, input requirements** and **cost** are the general determinants for practicality of an approach (Mertes et al. 2011). In essence, all enrichment approaches allow studies to be conducted at a multi – sample level by reducing sample complexity and focusing on genomic regions of interest.

2.3 Why to Pursue Target Enrichment?

Targeted approaches are an excellent adjunct when the surveillance of an entire genome is either not necessary or practical. Even though WGS is and will remain a gold standard for sequencing and re-sequencing purposes and to gain insights into all genomic regions, but for target-or trait-specific studies, approaches that provide improved sequencing depth of regions of interest are favored. Moreover, for complex genomes such as sugarcane, pine etc. WGS information is still not available, targeted sequencing represents a method to gain insights into those genomes. The array of benefits conferred by targeted approaches are listed in Fig. 2.2. Targeted approaches require less time and money to interrogate a broader gene pool with less

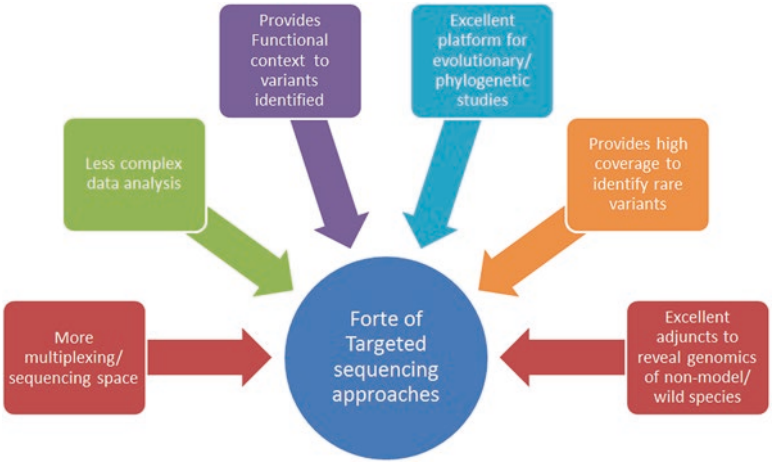

Fig. 2.2 Benefits conferred by targeted sequencing approaches

complex data analysis relative to re-sequencing of whole genomes of same number of genotypes.

Targeted sequencing permits deep sequencing of evolutionary conserved regions, which is beneficial for phylogenetic and systematic studies, as these do not require each and every base of the genome to be sequenced but rather rely on deep coverage of a sufficient number of informative loci to deduce broader and well resolved topologies. Relative to WGS, targeted approaches offers deep coverage and broader sampling of phylogenetically informative locus to determine topologies at different evolutionary time scale with increased resolution in a timely and cost effective manner. It limits the daunting challenges of complex data analysis and data storage associated at whole genome scale. Enrichment mode, size of population, evolutionary time scale, loci under consideration and the number, size and the properties of target are important parameters to be considered for addressing phylogenetic questions. Further, targeted and deep sequencing ensures phylogenetic accuracy as it selects for informative loci prior to sequencing rather than post sequencing; as later could cause systematic errors due to low quality- or unusable-data or data that cannot be effectively modeled. On the contrary, this could also lead to pre-enrichment bias, whereas WGS has certain amount of randomness built in it.

2.4 Strategies to Achieve Target Enrichment

Based on four strategies (Fig. 2.3), suites of methods are available to isolate specific genomic sequences from entire genome, either by removing unwanted components, i.e., target enrichment or by selecting desired targets, i.e., targeted pull down, for subsequent sequencing and downstream applications.

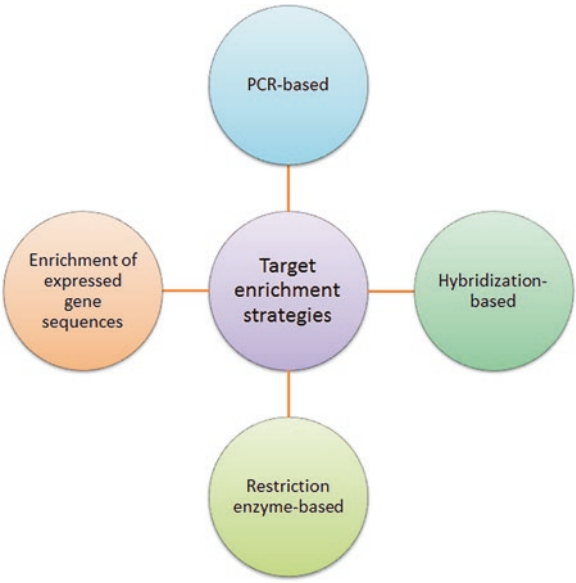

Fig. 2.3 Strategies to achieve target enrichment

2.4.1 PCR-Based Enrichment

Target enrichment via PCRs is the most straightforward technique to enrich for small sized targets from limited amount of input DNA. Based on the high degree of sequence conservation in the priming sites, the technique offers high target specificity; reproducibility and sequence uniformity, thus making PCR based enrichment a reference to compare different genome enrichment approaches. Long-range PCRs allows amplification of up to 50–100 kb of targets and is the most straightforward approach to accomplish enrichment of regions of interest. Further, if the target size lies within the range of sequence length offered by different NextGen sequencing chemistries, utilization of 5′ tailed primers in final amplification step allows one to bypass the requirement of shotgun library preparation prior to sequencing (Mertes et al. 2011). Thus, based on the amplicon length, either a direct sequencing of small amplicon or fragmentation of the long amplified DNA region via nebulization or sonication to prepare a library is carried out prior to sequencing.

PCR enrichment serves as an efficient technique for Sanger-based resequencing, but to harness the high throughput efficiency of NextGen engines, several alternatives to the uniplex PCR approaches have evolved and include modifications such as **multiplexing and microfluidic amplification of PCR products**. Multiplexing efficiently allows one to conduct enrichment of several targets in a single reaction and increases the throughput by running a number of multiplexed reactions in parallel. Microdroplet PCR is based on aqueous droplets (nanolitres-femtolitres in volume) serving as individual reactors/discrete PCR reactions and allows millions of distinct PCR reactions to occur per hour. An elegant alternative to this method is microfluidic amplification of PCR products utilizing microfluidic chips. Access Array

System (Fluidigm, San Franciso) and the Rain Dance system (Rain Dance technologies, Lexington, Massachusetts, USA) are commercially available platforms based on PCR amplification in microfluidic reactors followed by pooling of samples for multiplexed sequencing. **Access Array**™ system combines the library preparation for sequencing with amplicon generation by combining the sample specific tags and sequence specific primers during PCR. Amplicons are prepared via 4 primer tagging or multiplex primer tagging approach and for amplification of longer target regions, targets are split into multiple fragments with an overlap of at least 70 bp. In **4-primer tagging approach**, tagged target specific primers are combined with sample specific primers (contains barcodes and adaptor sequences) to produce up to 2304 unique amplicons using 48.48 dynamic Access Array Integrated Fluidic Circuit (IFCs), which could be further pooled for sequencing using Illumina. **Muliplex primer tagging** is a two-step process and can generate up to 480 amplicons per sample. The initial step is conducted on 48.48 IFC using sequence specific primers followed by amplicon harvesting and addition of sample specific barcodes and adaptor sequences with a 96- well plate to generate up to 23,040 unique products that could be pooled for further sequencing using Illumina (https://www.fluidigm.com).

Micro-droplet PCR approach by Rain Dance technologies involves preparation of 2 types of lipid encapsulated libraries, in which one library is composed of droplets containing unique primer pairs for different targets (Primer library) and second library consists of droplets of fragmented genomic DNA sample. Respective droplets are fused to generate a highly multiplexed emulsion PCR (Droplet PCR) and allows up to potentially 20,000 primer pairs to be used per reaction tube, followed by emulsion breaking to harvest the amplicons. Designing of a high quality primer library is critical to the success of the microdroplet PCR approach. Each primer pair (library element) is encapsulated to prevent interaction between primer pairs and different library elements are combined in equal number of droplets to prepare a final primer library. Microdroplet based PCR amplification is highly specific, sensitive and allows massively parallel uniplex enrichment of genomic subsets (Tewhey et al. 2009). Various advantages and limitations conferred by PCR based enrichment are depicted in Fig. 2.4.

PCR based enrichment has been extensively utilized for variant discovery as well as to characterize targets from nuclear genomes and plastomes and to infer phylogenetic relations and systematic studies. The strategy is reasonable for accessing only small genomic targets (e.g., <100 kbp) and the chloroplast genomes has served as the primary target till date, for example, plastome of genus pine was targeted using amplicon sequencing (Cronn et al. 2008; Parks et al. 2009; Njuguna et al. 2010). Uribe-Convers et al. (2014) amplified chloroplast genomes of 30 angiosperms and defined a set of 58 potentially universal primers using long range PCR in combination with NextGen Sequencing to deduce phylogenic relations. Durstewitz et al. (2010) analyzed SNPs from 100 EST amplicons in a panel of *B. napus* varieties and within its parental ancestral genomes i.e., *B. rapa* and *B. oleracea*. Kinoti et al. (2017) conducted amplicon NGS to explore variations of Prunus necrotic ring spot virus (PNRSV) from 53 infected trees to deduce a definition of PNRSV genetic strains using phylogeny and identity based methods.

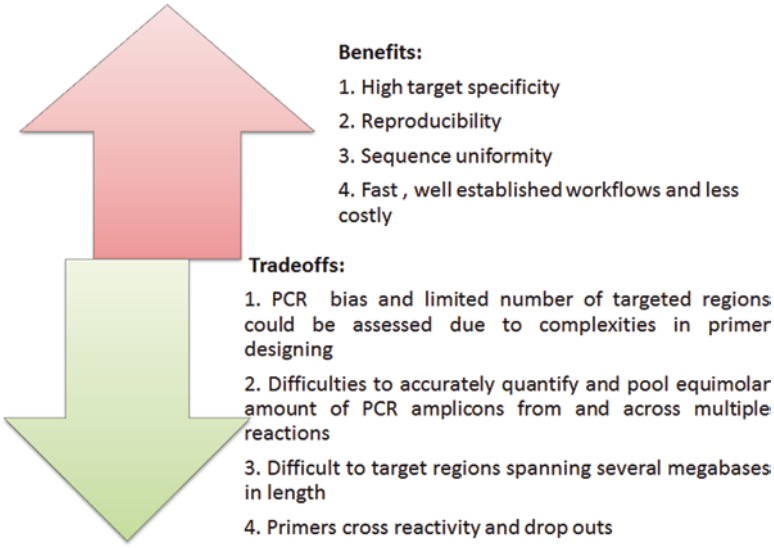

Fig. 2.4 Benefits and tradeoffs conferred by PCR based target enrichment

2.4.2 Hybridization-Based Enrichment

Utilization of pool of oligonucleiotide probes to facilitate identification and isolation of homologous, co-hybridizing targets have been exploited in various hybridization driven methodologies, such as Southern Blotting (Southern 1975), Microarrays (Hughes et al. 2001) as well as sequence capture approaches (Bashiardes et al. 2005). Hybridization based sequence capture could be aimed either for **target enrichment,** i.e., using probes complementary to regions of interest or **target depletion,** i.e., using probes to remove unwanted portions of the genome (Fu et al. 2010). Sequence capture could be approached in 2 ways, i.e., **array based and in-solution**, and the latter confers various technical improvements over the former (Fig. 2.5).

Array based hybridization utilizes probes bound onto fixed solid surface and targeted DNA fragments gets hybridized to immobilized probes and gets captured while non-specific DNA fragments are washed away. Array based enrichment provides good coverage and are less costly, but requires high amount of input DNA. **In-solution hybridization** utilizes free biotinylated probes which are allowed to hybridize to DNA library and the catch (Target-probe heteroduplexes) is separated from the non-specific fragments using streptavidin coated magnetic beads. In-solution based enrichment utilizes free probes and provides good recovery of probe-target hybrids. Common to both approaches, targeted DNA fragments are then eluted, amplified and subjected to sequencing.

While designing a hybridization based sequence capture assay, parameters such as input DNA quality and quantity, probe design, number of targets, GC ratio, coverage depth for each target (Zhou and Holliday 2012), expected enrichment

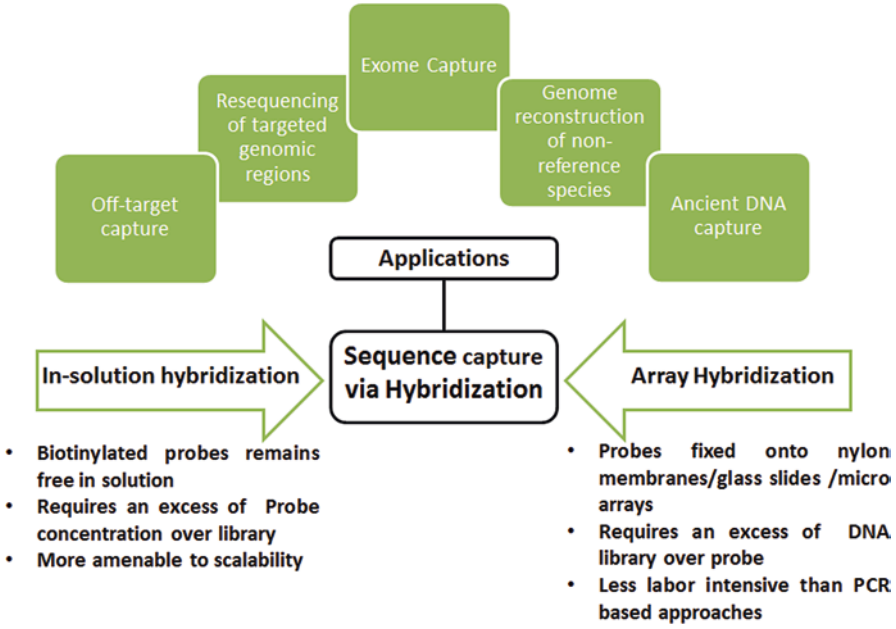

Fig. 2.5 Approaches and applications of hybridization based enrichment

efficiency, NGS technology used and biological system studied etc., needs to be addressed to drive a successful assay. Coverage depth is governed by multitude of factors such as probe specificity, genomic regions (conserved/unique) used for design of probe, genome size, homeology, heterozygosity, ploidy, presence of orthologs and paralogs genes etc. Single stranded RNA or dsDNA probes could be used as a bait to drive hybridization reaction, though utilization of ssRNA has several advantages, namely, higher affinity and melting temperature of RNA-DNA hybrids and absence of RNA probe complement prevents probe reannealing that deplete effective probe concentration (Sambrook and Rusell 2001) but RNA probes are in general less efficient than DNA probes. Further, the inclusion of blocking agents in a hybridization based enrichment is must and is required in excess amounts (Bashiardes et al. 2005) to serve two purposes: (a) to limit the extent of non specific or off-target capture, and (b) to prevent linking among targets i.e., secondary capture of fragments based on intrinsic repeat content. Secondary capture occurs when a target fragment hybridized to probe contains some repeat sequence that could potentially hybridize with any other repeat containing fragment. This necessitates the development of species specific blocking reagents to drive sequence capture experiments (Gnirke et al. 2009; Fu et al. 2010). To circumvent this, Fu et al. (2010), devised a blocker-free sequence capture strategy in maize, amenable to any plant species and defined as **Repeat subtraction-mediated sequence capture** (RSSC). They reported approximately 80–98% coverage of targeted regions and 1800–3000-fold enrichment accomplished in 2 capture stages. First capture intends to reduce

the repetitive regions of the genome using a repeat subtraction array, based on repeat content of the genome and second capture is based on target specific probes of capture array to enrich for desired fragments from the reduced complexity sample. Following which, capture fragments are eluted and amplified for downstream sequencing purposes. Further, to prevent sequencing of ineffectively enriched libraries containing off target- or repetitive sequence-captures, Klonowska et al. (2016) designed PCR based strategy incorporating **MLPA** (Multiplex ligation dependent probe amplification) probes and capillary electrophoresis to validate the enrichment efficiency of enriched library.

Hybridization-based sequence capture has several applications. Firstly, the capture could be used to **target resequencing of large genomic regions** using tiling probes to detect genomic variants and is commonly applied in Genome wide association studies (GWAS) to detect variation associated with a particular phenotype. Secondly, exon specific probes could be used to capture the most important genomic subset i.e., protein coding regions or **Exome capture** (described below). To further extend the scope of exome capture, a third application of hybrid capture, i.e., **off target capture** includes the captured fragments present outside of coding regions and potentially consists of UTRs, promoters, introns and intergenic region. Off target capture data provide valuable information on flanking sequences and thus could be a source of yet unidentified information and provide new insights for genome exploration. Fourthly, utilizing probes designed from PCR amplicons, ESTs, clone libraries, RNA-Seq or from orthologous regions of divergent genome, it is possible to extend the capture of targeted regions to non-reference species. Power of hybridization based sequence capture thus could be used for **genome reconstruction of non-reference** or wild relatives. Lastly, hybridization capture approaches could be extended to **ancient DNA** capture from samples from herbarium, museums and even to degraded DNA samples to deduce phylogeny relations precisely (Gasc et al. 2016).

Exome sequencing, i.e., power to sequence and enrich the complete protein coding region of the genome, as a major application to hybridization based enrichment is depicted in Fig. 2.6. Since exome enrichment targets the mRNA coding genomic fraction that eventually translates into phenotype, the approach thus restricts attention to the most informative part of genome. Exomes comprises high level of functional variants with less repeat content and constitute a relatively small fraction (1–2 Mb, depending on species) in comparison to whole genomes. It thus serves as an attractive alternative to access genomes of species intractable to whole genome sequencing efforts. Though exome analysis provides information onto unexpressed genes of a genome that did not form a part of a particular RNA-Seq experiment, it does not allow quantification of gene expression level.

After a first successful demonstration of exome capture studies in Maize by Fu et al. (2010) to enrich for 2.3 Mb region and 43 dispersed gene set and its extension to whole exome capture of maize (Liu et al. 2012b), exomes of number of crops has been explored including wheat (Saintenac et al. 2011; Winfield et al. 2012, 2016; Allen et al. 2013), soybean (Haun et al. 2011), barley (Mascher et al. 2013, 2014; Wendler et al. 2014; Pankin et al. 2014), black cotton wood (Zhou and Holliday

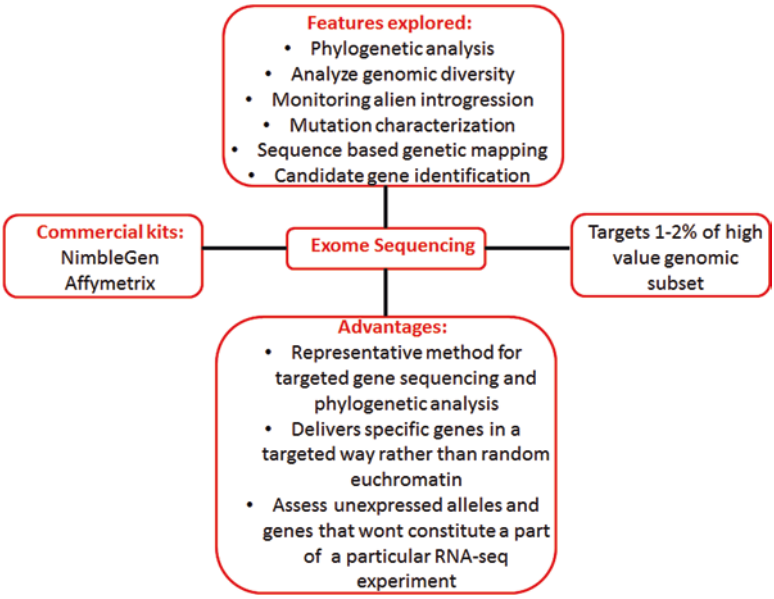

Fig. 2.6 Overview of exome sequencing, a major application of hybridization based sequence capture

2012), pine (Neves et al. 2014); eucalyptus (Dasgupta et al. 2015) and brassica (Galvao et al. 2012) etc. Cross capturing of exomes using probes from sorghum have been effectively applied to characterize sugarcane genotypes (Bundock et al. 2012) and lately, Song et al. (2016) captured exome of 12 different sugarcane accessions and provided a first report on saccharum haplotype based on 406 candidate genes. Exome capture has also been used successfully to characterize induced variation and identify gene function in soybean (Bolon et al. 2011), rice (Henry et al. 2014) and wheat (Gardiner et al. 2014, King et al. 2015, Krasileva et al. 2017). Exome capture of EMS mutagenized lines of wheat in combination with R gene enrichment sequencing led to successfully cloning of *Sr22* and *Sr25* stem rust resistance genes (Steuernagel et al. 2016). The resistance gene (NB-LRR gene i.e., Nucleiotide binding-site leucine-repeat rice gene) targeted enrichment and sequencing is referred to as **RenSeq** and has been successfully demonstrated to annotate the R genes in poorly as well as fully assembled genomes and also to identify variation for a pathogen resistance specific trait. Jupe et al. (2013) successfully applied RenSeq to a sequenced potato clone and reported an increased number of NB-LRRs and also identified markers co-segregating with resistance against *Phytophthora infestans*.

Among other utilities conferred by hybridization-based capture, its applications in context to **phylogenetic studies** are its forte. Deep coverage via targeted sequencing permits to incorporate more variation in probes to generate family wide probe sets to deduce well resolved topologies with strong bootstrap values (Jones and

Good 2016). Protein coding regions, i.e., exons are generally conserved, but at the same time harbor variability in third codon position, thus making them an excellent choice to design probes that will ensure for target specificity as well as their suitability to capture targets across diverse taxas. To infer phylogeny at moderate to deep evolutionary scale, exons are generally targeted while introns are targeted to infer phylogeny at or below the species level. Exome sequencing was successfully applied to deduce phylogeny among milkweed genotypes (Weitemier et al. 2014), Arecoideae tribe members (Comer et al. 2015), Asteraceae (Mandel et al. 2014) Medicago (de Sousa et al. 2014) and Hordeum (Brassac and Blattner 2015). Relative to other targeted approaches, hybridization based sequence capture offers additional advantages to infer phylogeny, such as,

(a) Restricts the capture and sequencing of contaminant DNA in paleontological samples
(b) Hybridization capture permits enrichment from degraded DNA samples obtained from herbariums/museums thus providing evolutionary insights in extinct as well as extant species (de Sousa et al. 2014)

Thus the wide scale of applicability of hybridization based approaches to target large scale genomic regions, i.e., insights into complex and large genomes of different angiosperms, population genomics and phylogenomics studies has been successfully demonstrated.

Advantages of Hybridization Based Sequence Capture

1. High degree of multiplexing to pool libraries of different samples.
2. Employment of longer probes permits hybridization tolerance for polymorphism within the capture probe region in comparison to approaches based on extension or ligation.
3. Allows better capture uniformity than enzymatic method (Turner et al. 2009).
4. Ability of short probes to capture larger targets as well as target enrichment from degraded samples that are not amenable to capture by PCR amplification.
5. No reference sequence required.
6. Hybridization based capture is scalable for both short discontinuous and long genomic targets and offers high reproducibility and less variance in target coverage (Gnirke et al. 2009; Harvey et al. 2013).

Limitations of Hybridization Based Sequence Capture

1. Cross hybridization between similar sequences results in non-specific capture, i.e., capture bias.
2. Cannot capture unique insertions of the target pool.
3. Initial capture design efforts and requires *a priori* knowledge to design probes.

2.4.3 Restriction Enzyme-Based Enrichment

Enrichment via restriction enzymes, chiefly aims to identify the most abundant markers across the genomes i.e., SNPs to facilitate development of highly dense genetic maps for downstream application such as map based cloning, association mapping etc. Different methods utilizing restriction enzymes based on genomic sub selection for targeted SNP discovery and genotyping are: Restriction-site-associated DNA tag sequencing (RAD-Seq) and modifications, Genotyping-by-Sequencing (GBS) and modifications and Genome reduction based on restriction site conservation sequencing (GR-RSC).

2.4.3.1 Restriction-Site Associated DNA Tags Sequencing (RAD-Seq)

First described by Miller et al. (2007a, b), RAD tag identification was based on microarray hybridization utilizing same subset of genomic regions across multiple samples to identify SNPs. Baird et al. (2008) modified the methodology and combined the RAD marker isolation and llumina sequencing of same genomic subsets across individuals. Briefly, the modified methodology involves, digestion of genomic DNA with a single restriction enzyme (Choice of enzyme dependent on genome size, GC content, coverage depth required and frequency of RAD tags across the genome), followed by adaptor ligation (P1). P1 adaptor comprises of Illumina sequencing primer, a unique barcode and forward PCR amplification priming site and gets ligated to the overhang heads produced by restriction digestion of genomic DNA. This is followed by pooling of PCR products, random shearing of fragments, size selection and ligation with adaptor P2. P2 adaptor is a Y shaped structure with divergent ends comprising of reverse complement of the PCR reverse priming site. Since the complementary sequence gets filled during the first round of forward elongation by primer present in P1 adaptor, inclusion of divergent Y shaped P2 adaptor ensures selective and robust enrichment amplification of only P1 adaptors ligated RAD tags (Fig. 2.7).

RAD tags allows identification of both dominant SNPs i.e., SNPs present within the restriction enzyme recognition site and co-dominant SNPs i.e., SNPs present within the tag but outside the restriction site. Different marker densities could be developed based on choice and number of restriction enzymes in a particular study and barcoding allows multiplexing of samples to assist fine mapping of genes. Moreover, the effective coupling of development of RAD tags and linkage mapping has been demonstrated for QTL analysis of reproductive traits in barley (Chutimanitsakun et al. 2011) and stem rust resistance in *Lolium perenne* (Pfender et al. 2011) and cultivated strawberry (Davik et al. 2015). RAD-Seq holds broad applications in *de novo* variant discovery, candidate gene identification and linkage mapping studies. The technology has been utilized for marker development in lolium (Pfender et al. 2011), sunflower (Pegadaraju et al. 2013), Cynara (Scaglione et al. 2012); Miscanthus (Slavov et al. 2014); for genetic mapping in *Lupinus augustifolius* (Yang et al. 2013); QTL mapping in perennial rye grass (Hegarty et al. 2013), barley (Chutimanitsakun et al. 2011) and for population structure analysis in chickpea (Varshney et al. 2013) etc. Xu et al. (2014) extended the applicability of

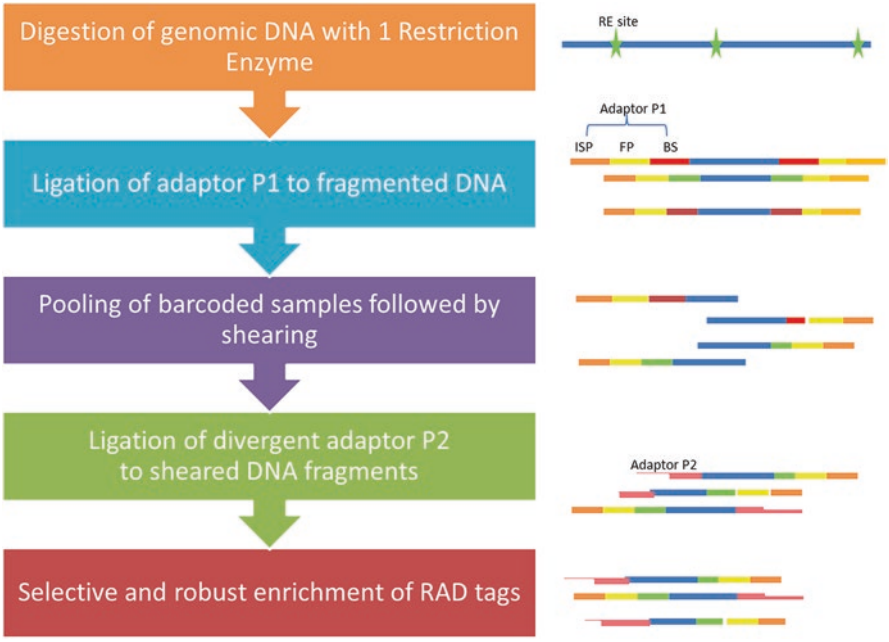

Fig. 2.7 Overview of development of restriction-site associated DNA (RAD) tags (RE- restriction enzyme site, ISP- Illumina specific sequencing primers, FP- PCR forward primer priming site, BS- barcode sequences)

RAD-Seq to population genomics studies of non model species, i.e., bottlegourd and generated sub-gene pools from 80 accessions associated with fruit shape and identified an orthologue of tomato fruit shape gene Ovate on LG7 of bottlegourd.

Several modifications to the original protocol are constantly being developed so as to facilitate multiplexing to decrease the input cost and to develop more effective and universal protocols for RAD tag development (Fig. 2.8 and Table 2.1).

ddRAD (Peterson et al. 2012) incorporates a double digestion of genomic DNA using two restriction enzymes, of which one enzyme is a frequent cutter and other is a rare cutter. This double digestion is coupled with precise size selection to allow robust amplification of selected loci and excludes random shearing of fragments. This method excludes fragments flanked by either very close or very distant restriction enzyme recognition site. This method offers greater flexibility, robustness, requires less amount of genomic DNA as starting material and is both time- and cost-efficient in comparison to the former method. ddRAD-Seq has been utilized to develop a linkage map of kiwifruit (Scaglione et al. 2012), cultivated peanut (Zhou et al. 2014) as well as in canola genotyping (Chen et al. 2013). Another modification by Van Orsouw et al. (2007) is named as **Complexity Reduction of Polymorphic Sequences (CRoPS™)** and is based on utilization of methylation sensitive restriction enzymes. This method combines the selectivity of AFLP™ (Amplified Fragment length Polymorphism) technology with high throughput NextGen

Fig. 2.8 RAD- Seq modifications [double digest RAD-Seq (ddRAD-Seq), Complexity Reduction of Polymorphic Sequences (CRoPS™), Multiplexed Shotgun sequencing (MSG), Improved 2b-RAD (I2b-RAD), Specific Locus Amplified fragments Sequencig (SLAF-Seq), RESTriction fragment SEQuencing (REST Seq), Rapture (RAD-capture)]

Table 2.1 Features of different RAD-Seq modification

Technique	Number of enzymes	Type of enzyme	Shearing of DNA	Size selection
RAD	1	Rare	+	+
ddRAD	2	Rare + frequent	–	+
ezRAD	1 or more	Frequent	–	+
REST Seq	2	Frequent	–	+
SLAF	2	Frequent	–	+
2bRAD/ I2bRAD	1	Frequent	–	–
MSG	1	Frequent	–	+
CRoPS	2	Rare + frequent	–	+
Rapture	1	Rare	+	+

sequencers. Since AFLP™ involves selective and robust amplification of restriction fragments from a pool of genomic DNA, the method offers a reduction in genome complexity by virtue of choice and nature of restriction enzymes to be included, multiplexing, no prior sequence information requirement and variable number of

selective bases that could be included in the primers during amplification. An approach similar to RAD that also incorporates the benefits of whole genome resequencing was developed by Andolfatto et al. (2011) as **Multiplexed Shotgun sequencing** (MSG). It involves restriction of genomic DNA by a frequent cutter enzyme in comparison to a rare cutter used in RAD-Seq and bypasses the shearing or repairing of DNA fragments. Ligation of a large number of fragments generated by a frequent cutter to a single set of barcoded adaptors, random orientation of fragments w.r.t., to sequencing direction, highly simplified protocol, time- and cost-efficient, small amount of input DNA requirements and no manual shearing requirements are some of the highlights of this method.

2b-RAD Seq method based on the generation of uniform length tags by virtue of type IIB restriction enzymes was developed by Wang et al. (2012) to genotype and characterize natural allelic variation and allows surveying of nearly all restriction enzyme sites and allowing marker intensity adjustments (Poland et al. 2012). Since 2b-RAD allows multiplexing by use of indexed primers in comparison to barcoded adaptors and pooling of samples during library preparation, 2b-RAD is demanding in terms of manpower and time. A much simpler, cost effective, fast and improved 2b-RAD (**I2b-RAD**) approach was reported by Guo et al. (2014) that incorporated the use of 12 barcoded adaptors and described the application potential of 2b-RAD tags for genetic mapping, QTL identification and other studies. Inclusion of double barcoding to permit high resolution and large scale genotyping, **Specific Locus Amplified fragments Sequencig** (SLAF Seq) was developed by Sun et al. (2013). Another modification to the traditional approach is **ezRAD** by Toonen et al. (2013) and involves the use of proprietary kits for adaptor ligation and further allows great flexibility in choice of restriction enzyme (single or combination) to fragment DNA in a desired size range, thus eliminating the use of separate adaptors for separate enzymes/sonication steps. **RESTriction fragment SEQuencing** (REST Seq) by Stolle and Moritz (2013) incorporates a two step digestion by 2 frequent cutters. Following digestion of genomic DNA with 1 frequent cutter, the fragments are adaptor ligated and subject to a second digestion by frequent cutter. Care should be taken in choosing the secondary enzyme for digestion as it should not be having any restriction site in the adaptor sequences. Following the digestion, the uncleaved fragments having adaptor sequences at both ends are recovered and subjected to sequencing.

Another approach added to the growing suite of RAD-Seq modification is recently developed by Ali et al. (2016) that physically separates RAD tags from the rest of the genome prior to the preparation of sequencing library and is described as **Rapture** (**R**AD-c**apture**) (Fig. 2.9). Similar to the procedure of Miller et al. (2007a, b), RAD tags are purified using streptavidin coated magnetic beads subsequent to ligation of fragmented genomic DNA with biotinylated RAD adaptors. These purified RAD tags, thus represents barcoded double stranded sheared DNA fragments which could be used as starting material for any library preparation protocol on any sequencing platform. Rapture protocol offers more flexibility for library preparation, excels at sample multiplexing, reduces clonality and is more cost effective than traditional protocol.

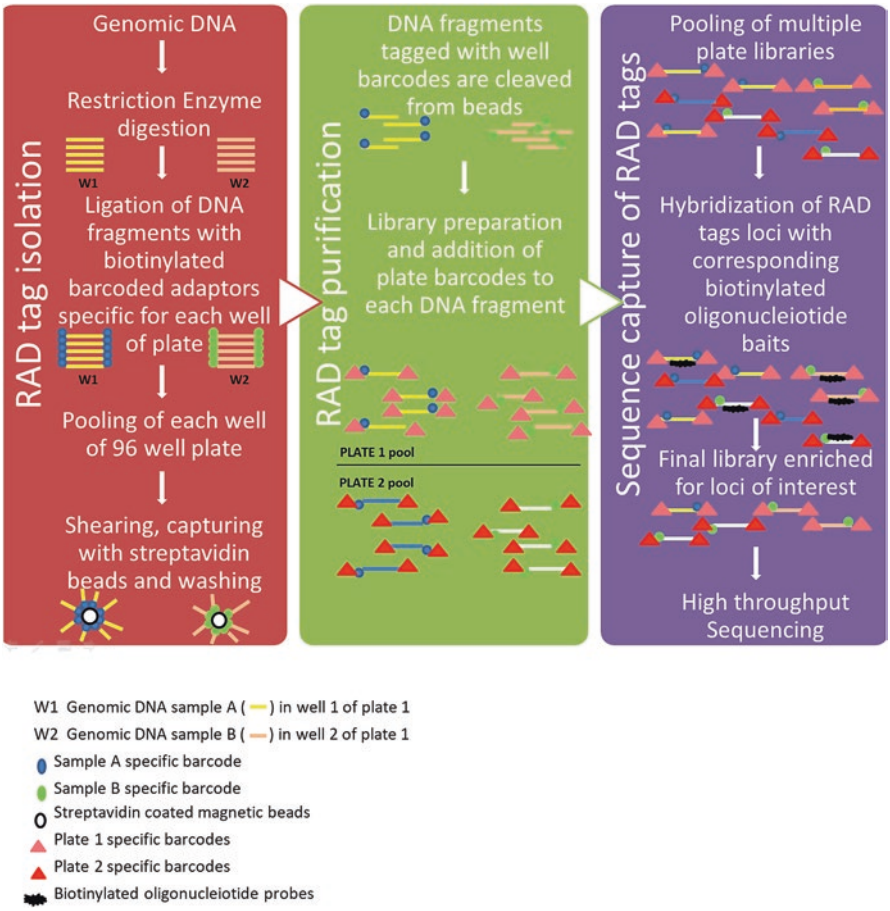

Fig. 2.9 Overview of rapture (RAD-capture) strategy

RAD-Seq offers a simple, timely and cost effective procedure for several applications and requires no prior knowledge. But the method also suffers from certain tradeoffs such as, (a) enrichment for anonymous loci, (b) not all the restriction sites in the entire genome are surveyed, (c) allele dropouts and PCR bias.

2.4.3.2 Genomic Reduction Based on Restriction Site Conservation (GR-RSC)

Developed by Maughan et al. (2009), this method of genomic reduction is based on double digestion of genomic DNA using a 4 bp (frequent cutter) and 6 bp (rare cutter) cutter, followed by adaptor ligation. Adaptor corresponding to a restriction site of 6 bp cutter is labeled with 5'biotin molecule and an unlabeled adaptor corresponds to a restriction site of 4 bp cutter. Genomic reduction is ensured using biotin-streptavidin paramagnetic beads that captures only the labeled DNA fragments.

This is then followed by pooling of different DNA fragments, size selection and subsequent sequencing using high throughput sequencers. Similar to RAD-Seq, GR-RSC procedure does not require any prior knowledge and offers a simple platform to identify variants for large scale genotyping. GR-RSC developed markers exhibit an even distribution across the chromosomes (Maughan et al. 2009, Byers et al. 2012). But the procedure does not target specific variation and enriches for anonymous loci. The procedure has been successfully demonstrated in Arabidopsis, Amaranthus (Maughan et al. 2009, 2010), cotton (Ashrafi et al. 2012; Byers et al. 2012).

2.4.3.3 Genotyping-by-Sequencing (GBS)

Elshire et al. (2011) developed a technically simple, cost effective and highly multiplexed approach utilizing high throughput NGS techniques for sequencing of genomic subsets, known as **Elshire-GBS**. Reduction in genome complexity utilizing restriction enzymes offers a quick, easy, specific and reproducible method that could target the regions of genomes inaccessible by sequence capture approaches. The efficacy of restriction enzymes to reduce genome complexity was actually demonstrated by Baird et al. (2008) through development of RAD tags for numerous downstream applications. GBS methodology utilizes methylation sensitive restriction enzymes to enrich for a low copy fraction of the genome with reduction for repetitive regions. Both GBS and RAD-Seq target regions flanking the restriction site but the library preparation to perform GBS is much more simplified in comparison to RAD as the former requires less DNA, avoids random shearing and size selection and gets completed in 2 steps with subsequent amplification of pooled library. The basic methodology of Elshire-GBS is shown in Fig. 2.10. GBS was also successfully extended to impart better genomic resolution when coupled to whole genome resequencing (Huang et al. 2009).

Elshire-GBS is based on a single restriction enzyme to enrich for genomic subsets between the restriction sites. This technique was successfully extended to 2 enzyme systems (rare cutter + common cutter) by Poland et al. (2012) and is known as **2-Enzyme GBS**. This approach results in generation of more uniform libraries for sequencing in which all the fragments are ligated by adaptor 1 on one end and adaptor 2 on the other end. The incorporation of Y shaped adaptor 2 along with digestion of genomic DNA by 2 enzymes simplifies library quantification prior to sequencing.

Choice of restriction enzyme employed in GBS depend on factors such as: number of markers to be developed, multiplexing level, type of study and whether genic SNPs are preferred or not. Since the restriction enzymes used influences the type and number of fragments represented in a GBS library, Pootakham et al. (2016) evaluated multiple enzyme combinations and reported that usage of 2 methylation sensitive enzymes in oil palm preferentially enriches for genic SNPs with library preparation protocol without any size selection step and allows for high degree of multiplexing as it yields comparatively less number of scorable SNPs, while incorporation of methylation insensitive enzymes requires an additional step of size fractionation of fragments to improve read depth and enriches for intergenic SNPs. GBS

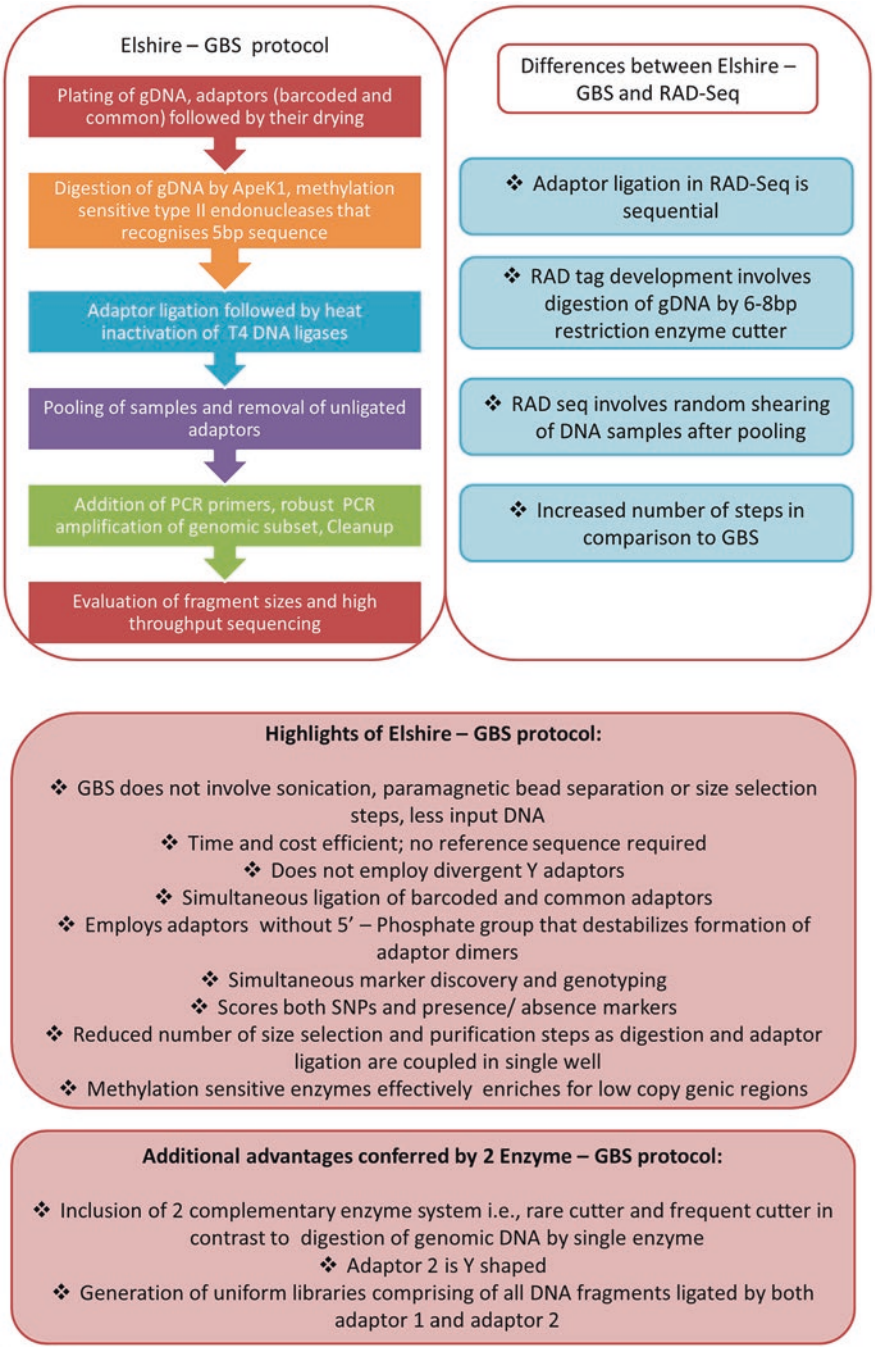

Fig. 2.10 Overview of genotyping by sequencing

and its modifications successfully couple genomic complexity reduction utilizing restriction enzymes with multiplexing and high throughput sequencing to generate a large amount of valuable data with little input cost per data point and in time proficient manner. It excludes the precondition of identification and validation of polymorphism and rather combines marker discovery with genotyping, making it an ideal breeding strategy for utilization in any population/species, including even those without reference genome or with availability of aforementioned polymorphism information (Schnable et al. 2013).

Flexibility, low cost, less ascertainment bias of markers, GEBV (Genomic estimated breeding values) prediction value accuracy constitutes the forte for the utility of GBS in genomic selection studies (Bhat et al. 2016). GBS has been extensively utilized for marker development, breeding applications, association studies, genomic selection, gene/QTL mapping, population structure, Bulked Segregant Analysis (BSA) etc.

GBS is successfully demonstrated for above mentioned applications in a number of plant species such as barley (Poland et al. 2012), grapevine (Myles et al. 2010), maize (Tian et al. 2011; Gore et al. 2009), switch grass (Lu et al. 2013), soybean (Sonah et al. 2013), black currant (Russell et al. 2014), potato (Uitdewilligen et al. 2013) canola (Raman et al. 2014), rubber tree (Pootakham et al. 2015), cotton (Islam et al. 2015), wheat (Gao et al. 2015, Michel et al. 2016, Arruda et al. 2016) and the list is still continuing.

2.4.4 Enrichment of Expressed Genome Sequences/ Transcriptome Sequencing/RNA-Seq

Since the transcribed portions of the genomes range from approximately 25% of the compact and gene dense angiosperms genomes to 1% of the repeat rich genomes of crucifers, capturing the transcriptome is an efficient genome reduction strategy (Rabinowicz et al. 2005). Considering the fact that the coding portion is particularly enriched for variants and harbors less of the repetitive elements, RNASeq allows direct sequencing of cDNA derived from RNA to quantify and monitor gene expression profiles. RNA-Seq, provides information onto structure of gene and transcript, polymorphism within exons, introns and flanking non-coding regions, quantification of gene expression information onto developmentally and environmentally regulated genes and also aids in detection of novel isoforms and transcripts. RNA Seq begins with the isolation of high quality total RNA from the tissues. Since rRNA (ribosomal RNA) constitute a major fraction of total RNA, isolated RNA is enriched for protein coding polyA tailed mRNA (major proportion of expressed portion of genome) based on oligod(T) selection. Different approaches are available for the selective enrichment of mRNA. For example, enrichment of non-polyA mRNA along with polyA mRNA fraction by depleting of rRNA using hybridization based probes, provided by different manufacturers, such as RiboMinus (Invitrogen) or Ribo-Zero (Epicentre). Generalized procedure for transcriptome sequencing involves fragmentation of enriched RNA fraction, followed by first strand cDNA

synthesis, second strand DNA synthesis and platform specific adaptor ligation to create a double strand cDNA library, PCR enrichment and subsequent sequencing and downstream analysis.

The strength of transcriptome sequencing lies in the fact that it requires no prior genomic information in comparison to hybridization based methods or PCR based methods that require *a priori* information to design probes or primers and is free from the enzymatic bias inherent to the restriction enzyme based methods. It is thus a powerful tool to analyze global gene level expression differences, but is limited by the bias associated with transcript abundance and is also dependent on tissue and stage at which RNA-Seq is conducted.

RNA-Seq is used to interrogate genome level, gene level and transcriptome level variation. For species lacking the availability of reference genome assemblies, transcriptome assembly serves as a medium to gain insights into the genome, transcriptome and metabolome of many important crops such as rye, switch grass, alfalafa, hevea and several medicinal plants such as Lemongrass (Meena et al. 2016). RNA-Seq has been deployed to design molecular markers in a cost efficient manner for crop plants such as potato (Hamilton et al. 2011), tomato (Hamilton et al. 2012), alfalfa (Yang et al. 2011), guar (Tanwar et al. 2017), Brassica (Wei et al. 2016, Teng et al. 2017) as well as perennial forage grasses such as Hemarthria (Huang et al. 2016) etc.

Combination of RNA-Seq with BSA analysis (BSR-Seq) has been effectively demonstrated in maize (Liu et al. 2012a) to identify the genomic position of glossy gene 3 (*gl3*) locus encoding for Myb transcription factor using mutant and non-mutant pools of *gl3* locus. RNASeq is also useful for expression QTL (eQTL) studies to quantify the allele specific expression and variation, splice form variation and to evaluate molecular markers (Majewski and Pastinen 2011; Gan et al. 2011). Ability of RNA-Seq to evaluate polymorphism at both genome and transcriptome (expression) level make it a useful tool for GWAS studies (Delker and Quint 2011; Li et al. 2013). Transcriptomics based GWAS is designated as Associative transcriptomics and utilizes sequence- and transcript abundance- variation to identify association with trait variation (Harper et al. 2012) and has been recently demonstrated successfully in *Brassica napus* seeds in relation to erucic acid content and tocopherol composition (Havlickova et al. 2018).

2.4.5 Other Reduced Representation Sequencing Techniques

DNA methylation of Cytosine at 5′C is mediated through three pathways (Fig. 2.11) and is a highly specific phenomenon that helps to regulate gene expression, chromatin structure, transgene silencing, paramutations, genomic imprinting, transposon activity, repression in recombination as well as rapid epigenetic adaptation in response to external stimuli or heritable phenomena. Genome wide studies in plants have reported 5–25% of cytosines being methylated (Zemach et al. 2010; Cokus et al. 2008). Methylation of gene bodies has been correlated with the medium to high expression of constitutively expressed genes (Zhang et al. 2006; Zilberman and

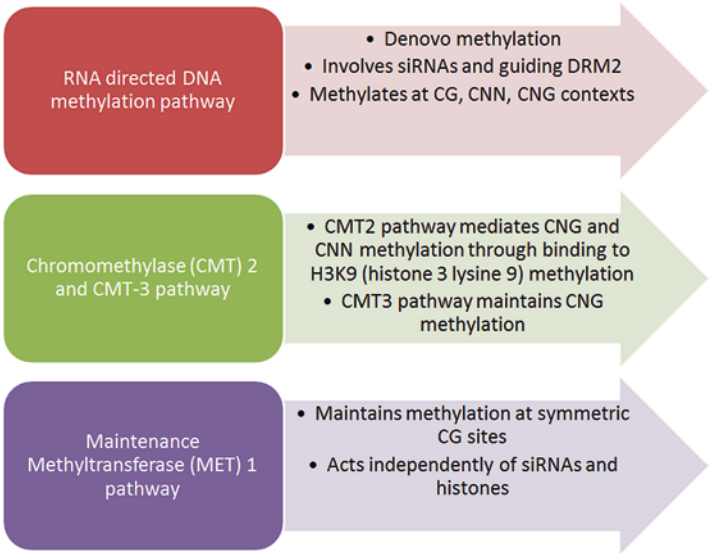

Fig. 2.11 Different pathways contributing to genomic DNA methylation

Henikoff 2007) while methylation of promoters tends to repress the expression (Zhang et al. 2006). Preferential methylation of plant genomes occurs at CG, CNN and CNG sequences in repetitive regions, heterochromatic region and inactivated transposons whereas euchromatin, transcribed regions and other low copy sequences are rarely methylated or their methylation is limited at 5′ or 3′ ends. This differential plant genome and epigenome landscape carved a path to devise strategies, namely, high C_0t selection (HC), Methylation filtration (MF), and bisulfite sequencing to filter the gene enriched sequences from large repetitive plant genomes.

2.4.5.1 High C_0t Selection

High C_0t (product of DNA concentration i.e., C_0 and reassociation time i.e., t in seconds) sequencing was the first methodology devised to separate the low copy genic sequences and repetitive fraction of genome based on rapid renaturation kinetics of repetitive sequences from completely denatured genome (Fig. 2.12). Basically, the technology relies on the renaturation rate of a sequence proportional to its frequency (relative abundance) in the genome (Peterson et al. 2002), i.e., abundantly available sequences associates with their complementary sequence faster in comparison to low copy sequences. C_0t experiment is characterized by fastest renaturation of most abundant repetitive elements and slowest renaturation of low copy number genic sequences of a fully denatured genome. The technique serves to isolate the low copy or high C_0t fraction of genomic DNA. It has been used successfully in maize (Whitelaw et al. 2003; Yuan et al. 2002) and sorghum (Peterson et al. 2002) to enrich for gene sequences.

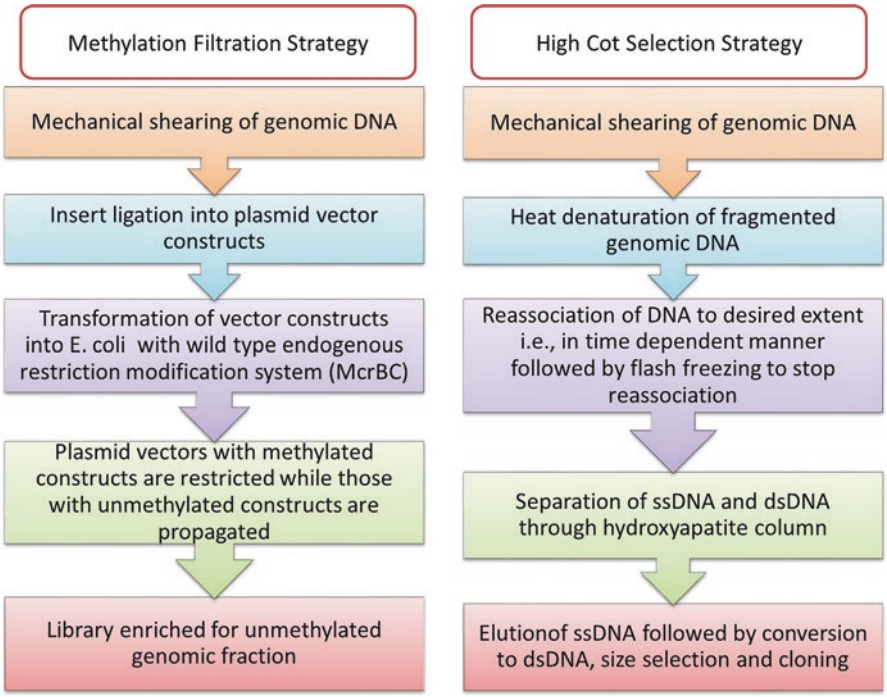

Fig. 2.12 Overview of methylation filtration and high C_0t selection strategy as reduced representation techniques (ss: single-stranded; ds: double-stranded)

2.4.5.2 Methylation Filtration (MF)

MF (Fig. 2.12) exploits differential methylation patterns across plant genomes to isolate genic sequences (non-methylated) from methylated sequences associated with repetitive elements utilizing bacterial methylation dependent restriction system (Rabinowicz et al. 1999; Sutherland et al. 1992). MF is based on small insert genomic libraries constructed in *E. coli* hosts with 5mC restriction system, i.e., McrBC (modified cytosine restriction system) that eliminate all clones possessing methylated repetitive DNA elements and to preserve, propagate and enrich the resulting libraries for genic sequences. A pilot study conducted by Rabinowicz et al. (1999) yielded a sixfold enriched library of maize genes in comparison to its WGS library.

MF has been exploited by Palmer et al. (2003) to characterize 100,000 hypomethylated sequences from 5–7-fold gene enriched library of maize genome as well as in sorghum (Bedell et al. 2005). Further success of MF as a gene enrichment technique has been demonstrated in a broad range of plant species, including monocot (rice, wheat, sorghum, etc.), dicots (soybean, Arabidopsis, cotton, tomato, etc.) as well as non-angiosperms such as mosses, ferns and pines were subjected to gene enrichment using MF to reveal enrichment of hypomethylated fractions for genes

and correlation between genome sizes and degree of gene enrichment (Rabinowicz et al. 2005). Grativol et al. (2014) compared the efficacy of MF for gene enrichment in methyl-filtered and unfiltered library, reported an increase in coverage of genic regions of monoploid sugarcane by 134X in MF library and generated a robust assembly that could be potentially used as a reference representing unigene dataset.

2.4.5.3 Reduced Representation Bisulfite Sequencing

Bisulfite sequencing (Clark et al. 2006) marks a significant role in characterizing complex and diverse plant DNA methylation patterns on a genome wide scale. Both cell- and tissue- specific methylation patterns have been reported in plants. Evaluation of cytosine methylation at the nucleotide-level involves the treatment of randomly sheared genomic DNA with bisulfite followed by NGS and is termed as whole genome bisulfite sequencing (WGBS). While methylation insensitive endonuclease restriction (used to avoid low cutting efficiency at restriction sites due to methylation) of genomic DNA to enrich for CpG regions of genomes, followed by size selection generates specific genomic fragments for subsequent bisulfite treatment and sequencing. Thus, it provides higher coverage of genomic subset at low sequencing costs relative to WGBS and is termed as reduced representation bisulfite sequencing (RRBS) (Meissner et al. 2005). The generalized procedure is described briefly in Fig. 2.13. Sodium bisulfite does not affect the 5-methyl cytosine whereas it efficiently deaminates the unmethylated cytosine to uracil. Thus, subsequent amplification and sequencing results in cytosine base calling wherever methylation was present and thymine base calling in the absence of methylation.

Several modifications to the original protocol of Meissner et al. (2005) has been developed. Targeted sequencing comprising of capturing of desired genomic regions prior to bisulfite treatment and sequencing has also been proposed (Lee et al. 2011) but requires an access to a well annotated and complete genome assembly to design probes specific to the desired genomic regions. Since the use of single restriction enzyme (as in the original protocol) has an access to a limited number of cytosine positions, subsequent modifications incorporates the use of more than one restriction endonuclease. Hsu et al. (2017) developed and demonstrated the first application of ROI (region of interest) directed RRBS to characterize role of methylation in vegetative to reproductive transition in maize by comparing tassels and shoot epigenomes. A pipeline was designed for *in silico* identification of restriction enzymes and subsequent optimization of fragment sizes to ROI. The pipeline (available at https://gitlab.com/fmhsu0114/maize_RRBS/tree/master), thus identifies suitable candidate restriction enzymes by taking into account size range of selected fragments, and their enrichment degree for ROIs. The enzymes and size range of fragments were decided when the ROIs were clearly enriched. Different genomes and ROIs necessitates selection of different candidate enzymes.

Another modification by Schmidt et al. (2017), referred to as plant-RRBS is a simple, cost effective, high- throughput methylome profiling platform. Briefly, the methodology involves double digestion of genomic DNA (combination of endonucleases chosen are first tested *in silico* for their cutting frequencies), followed by

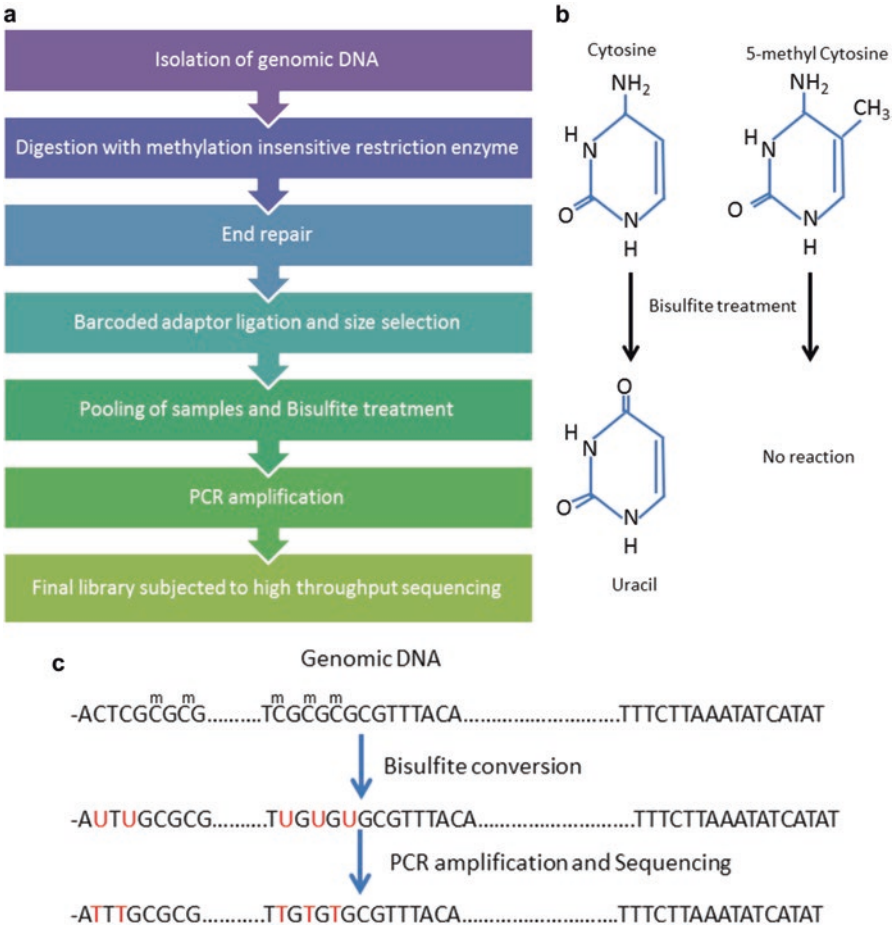

Fig. 2.13 Overview of reduced representation of bisulfite sequencing technique

end-repairing, adaptor ligation, bisulfite conversion, PCR amplification and sequencing of enriched library to provide insight into genome methylation pattern at a broad scale. RRBS has been done successfully to study methylome of a number of plant species such as in *B. rapa* subgenomes to gain insight into the role of epigenetic modifications in polyploid genome evolution (Chen et al. 2015) and to study promoters of Quercus genome in response to temperature regimes (Gugger et al. 2016).

2.4.6 Conclusion and Future Prospects

The utility of targeted enrichment strategies is a function of 4 parameters, i.e., level of sample multiplexing, required sequencing coverage, cost per sample and targeted genomic coverage. These approaches were initially developed to serve as an aide to

WGS approaches for affordable dissection of sub-genomic regions of complex genomes with simplified data interpretation and to generate resources for non-reference species for which WGS still poses a big challenge in terms of efficient and accurate genome assembly. But, targeted approaches have created a niche for themselves for their utility in a range of biological studies, starting from discovery of natural and induced polymorphism to efficient characterization of plant gene space, genetic map construction, QTL mapping, candidate gene identification and to resolve phylogenetic topologies. Moreover, queries pertaining to comparative genomic- or evolutionary- studies require analysis of a larger number of individuals rather than whole genome coverage, and are thus better solved through targeted approaches.

WGS will definitely become universally accessible in the near future as it offers more consistent genome coverage, more polymorphism, uniformity among sequencing quality parameters, absence of any reference sequence bias as generated by probes usage and will always remain a gold standard for genome sequencing purposes, but it is not possible and practical to resequence complete genomes of entire mapping populations/multiple accessions/cultivars. Thus, targeted approaches are not transitionary and are bound to stay as these have carved their own niche in solving biological riddles that do not require interrogation of whole genomes, especially for evolutionary and ecological research.

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Genomic Intervention in Wheat Improvement

3

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Abstract

Common wheat is the second most important cereal crop after rice worldwide. Hexaploidy nature of wheat genome makes it the model crop for the study of allopolyploid genomes with highly repetitive sequences. Conventional approaches of genome sequencing proved to be very tedious and time consuming for allohexaploid wheat. Therefore, with the advancement in the latest next generation sequencing technologies led IWGC to precisely map the wheat genome (~17 Gb). This genome sequence opens new avenues for functional characterization of genes which is the need of the hour for devising new strategies for wheat improvement. Here, we discuss the genome sequencing technologies, functional and comparative genomics of wheat to bridge the gap between genotype and phenotype.

Keywords

Wheat · Genome sequencing · Functional genomics · Comparative genomics

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3.1 Introduction

Triticum aestivum L. (bread or common wheat) is the second most important cereal crop that serves the purpose of satisfying hunger and nutritional security for more than 4.5 billion people in the world (IIWBR Vision 2050). Cultivation and domestication of wheat crop opened the avenues for prosperity of agriculture and flourishing the societal developments.

Wheat evolved approximately 10,000 years ago in a process called hybridization between cultivated tetraploid emmer (*T. dicoccum*, AABB) and diploid goat grass (*Aegilops tauschii*, DD) (Tanno and Willcox 2006) (Fig. 3.1).

With >620 million metric tons global wheat production India still holds second position after China (http://www.fao.org/3/a-i4691e.pdf). In the context of ever-increasing world population, which is expected to be ~9 billion by 2050, there is an ardent need to augment wheat production using genetic improvements to satisfy the food demands.

This herculean task has been greatly eased by advancements made in the field of next-generation sequencing (NGS) technologies. These have revolutionized the wheat functional genomics studies and has laid way for rapid forward genetics studies (Bevan et al. 2017).

The common wheat genome size is very large (~17 Gb) in comparison to other major crops, with each sub genome being approximately 5.5 Gb. The wheat genome (~80%) is chiefly made up of repetitive sequences (Marcussen et al. 2014). Owing to its large size, accurate sequencing and assembly of the entire wheat genome was very challenging task.

Recent advancements in experimental approaches, resources, and computational analysis tools have eased the identification of new genes that can be utilized in wheat breeding. In this chapter, we focused on recent progress made in the area of

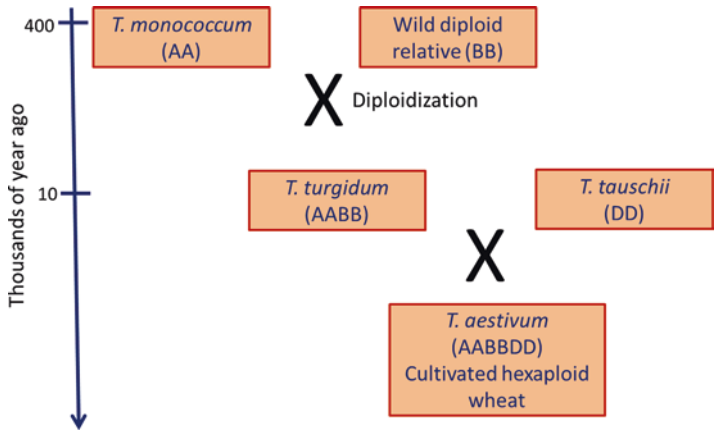

Fig. 3.1 Evolutionary path of modern cultivated hexaploid wheat

wheat genomics vis-à-vis its significant implication in improving wheat performance.

Furthermore, we highlighted the future strategies for genetic mapping of wheat and new resources for discovering the genetic variation. Identification of gene function will significantly assist in wheat improvement programmes.

3.2 Status and Opportunities of Wheat Genome Sequencing Program

International Wheat Genome Sequencing Consortium (IWGSC) was constituted in 2005 to uncover the intricate wheat genome in order to accelerate wheat improvement programme worldwide. Owing to the complexity of wheat hexaploid nature, all the chromosomes (21) of wheat landrace Chinese Spring were sorted by flow cytometry and were used for preparing BAC libraries and physical maps.

The initial task of chromosome sorting, DNA isolation and BAC library construction for each chromosome arm were taken by Prof. Jaroslav Dolezel at the Institute of Experimental Botany in the Czech Republic. The prepared BAC libraries were than subsequently assigned to different laboratories of the IWGSC for physical map construction and BAC sequencing.

Team efforts led to first successful sorting and sequencing of chromosome 3B (774 megabases (Mb) 5326 protein-coding genes) despite its large size (Paux et al. 2008). Till now all the chromosomes of Chinese spring have been sorted and physical maps have been generated for these chromosomes (http://www.wheatgenome.org/Projects/IWGSC-Bread-Wheat-Projects). Sequences of many chromosomes or parts including 1AS, 1BS, 3DS, 5DS, 7DS, 1AL,1BL, 4A, 5A, 6A, 6B, and 7B are available publicly (Hernandez et al. 2012; Breen et al. 2013). Chromosome-based draft sequence of Chinese Spring was published after 2 years of the first wheat genome release by IWGSC (2014).

Furthermore, IWGSC (2018) released a fully annotated reference genome sequence which could be utilized by the molecular breeders for exploring the genetically complex yield and quality parameters with stable crop yield.Flow cytometry based chromosome sorting and chromosome-based shotgun sequencing based on Illumina technology were exploited to yield 10.2 Gb of genome sequence. Intra- and inter-specific comparisons showed abundant gene losses and duplication indicating that the wheat genome was evolutionarily dynamic (IWGSC 2014). Clavijo et al., in 2017 generated new assembly of Chinese Spring representing >78% of the genome which was higher than the proportion (~49%) generated by IWGSC. For this they relied on precisely sized mate-pair libraries and an optimized algorithm. The final sequence of wheat genome was compiled using combined approach of next generation (short Illumina reads) and third generation sequencing data (long Pacific Biosciences reads).

The >15 Gb of final assembly that represented >90% of the Chinese Spring genome was created and is the most complete wheat genome sequence published yet (Chin et al. 2016). Recently, IWGSC announced that they have completed a

Table 3.1 Summary of hexaploid wheat sequencing programme of IWGSC (2014)

Sr. No.	Parameters	Features
1.	Cultivars	*Triticum aestivum* cv. 'Chinese Spring'; *T. turgidum* cv. *Strongfield; T. turgidum* cv. *Cappelli*; *T. urartu*; *Aegilops speltoides*
2.	Sequencing platform	Illumina HiSeq 2000 and Genome Analyser IIx
3.	Sequencing approach	Whole genome shotgun-next generation sequencing (WGS-NGS)
4.	Nucleic acid sequence	Nuclear DNA and messenger RNA
5.	Insert size of DNA libraries	500 bp
6.	Genome assembly software	Assembly By Short Sequences (ABySS)
7.	Transposons	Approximately 80% of the genome
8.	Draft genome size	17 Gb
9.	Total genes	124,201
10.	Reference release	IWGSC RefSeq v1.0 assembly

high-quality sequence of Chinese Spring (IWGSC v1.0) and released the genomic data for public access (http://www.wheatgenome.org/News/Latest-news/RefSeq-v1.0-URGI) (Table 3.1).

In addition to sequencing of Chinese Spring at the genome and single chromosome level, wild emmer wheat (the tetraploid ancestor of common wheat) was sequenced by Avni et al. (2017). This was obtained by using whole genome shotgun sequencing of various insert-size libraries that contributed to about 10 Gb sequence of wild emmer genome.

3.3 Journey of Genome Sequencing and Assembly Technologies

Whole genome or single chromosome arm were used in earlier genome sequencing technologies. The most recently used techniques were second generation sequencing combined with whole genome shotgun or BAC-by-BAC strategies. The major drawback of these technologies was that they generated short read-lengths (<300 bp) and provided biased genome coverage that resulted into fragmented and incomplete genome assemblies especially in complex repeat structures genomic regions.

Subsequently, a new sequencing technique known as SMRT (single molecule real-time sequencing technology by Pacific Biosciences, USA) was introduced which yielded significantly longer sequence reads (up to 40–50 kb). This type of read length enabled production of genome sequences with characteristic fewer gaps and longer contigs even for complex genomes (Ferrarini et al. 2013). Apat from this BioNano genome mapping and 10× Genomics linked reads, are other

next generation mapping technologies which are increasingly being applied in genome sequencing to produce high quality assemblies (Rasekh et al. 2017). Nevertheless, Hi-C platform can be deployed to determine the 3-D style of chromosomes, validate genome assembly and scaffold order to elucidate chromatin structure (Lieberman-Aiden et al. 2009). NRGene (NesZiona, Isreal) introduced the DenovoMAGIC2 assembler for complex genomes like that of wheat. It facilitated assembly of Illumina short reads into larger scaffolds with N50 up to several Mb (Avni et al. 2017).

3.4 Functional Genomics in Wheat

Functional genomics utilizes the functional characterization of genes. Hexaploid and tetraploid wheat lines presents significant complexity for transgenic research as they possess low transformation efficiencies and resulting high transformation costs. These complexities have limited the use of transgenic approach to Bobwhite and Fielder wheat lines. Recently, Ishida and coworkers (2014) have devised 'Purewheat' Agrobacterium transformation procedure from Japan Tobacco. This procedure has successfully improved transformation efficiencies up to 30–50% amongst modern commercial hexaploid and tetraploid cultivars (Richardson et al. 2014). This breakthrough could be utilized for functional characterization of wheat genes at an unparalleled scale.

The polyploid nature of wheat offers the ability to simultaneously down-regulate multiple homoeologous genes using RNA interference (RNAi) and has been a good option for functional genomics in polyploid wheat (Fu et al. 2007). Due to highest homoeologous genomes similarity single ~21 nt based RNAi construct can be used to silence multiple homoeologues.

These characteristics of RNAi has enabled its wider application to study a range of biological processes such as senescence, disease resistance, seed development and meiosis across organs (anthers, leaves, grains) (Travella et al. 2006; Uauy et al. 2006; Gil-Humanes et al. 2014).

A new member of SWI/SNF factors SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A, member 3-like 3 (SMARCA3L3) is targeted by tae-miR1127a. Its function is to assist in carefully regulated progression of meiosis in male gametophytes of wheat (Sun et al. 2018). The high genome sequence similarity engrossed innovative genome editing technologies such as transcription activator-like effector nuclease (TALEN; Moscou and Bogdanove 2009; Boch et al. 2009) and clustered, regularly interspaced, short palindromic repeats (CRISPR-Cas9; Cong et al. 2013). Wang et al. (2014) demonstrated that a single CRISPR/Cas9 and/or TALEN construct can generate targeted mutations across multiple homoeologues of the Mildew-Resistance Locus O (*Mlo*) gene and that these modifications can be inherited to the next generation (Wang et al. 2014).

The authors generated targeted gene insertions at a specific locus using non-homologous end joining at double-strand breaks caused by TALENs. These advanced genome editing technologies will aid in wheat functional genomics,

especially when leveraging the advances in genome sequencing and the reported tenfold increment in transformation efficiency. EMS mutagenesis and gene editing revealed inter-cultivar differences and additivity in the contribution of TaGw2 homoeologues to wheat grain weight and size (Wang et al. 2018). Plants hauling single copy nonsense mutations in different genomes showed varied levels of GS/TGW increase an average of 5.5% while for double mutants had on average 12.1% higher TGW as that of wild type lines. The highest enhancement in GS and TGW was shown for triple mutants of both cultivars, with increases in 16.3% (edited) and 20.7% (TILLING) in TGW.

Chemical and radiation-based mutagenesis in wheat breeding is widely accepted and known technique. Functional complementation between homoeologues has limited the use of mutagenesis in forward genetic screens especially single-copy disease tolerant genes (Feuillet et al. 2003; Periyannan et al. 2013). Use of the 'Targeting Induced Local Lesions in Genomes' (TILLING) approach (McCallum et al. 2000) provided a new way to analyze structured mutant populations for changes in a candidate gene. Here, the functional complementation between multiple homoeologues allowed wheat to endure more than tenfold higher mutation rates compared to related diploid species. This alters the range of allelic variants that can be obtained in wheat. For example, screening of only 1500 individuals in a hexaploid TILLING population should yield >65 mutant alleles for a 1.5 kb target, but only 6–7 mutant alleles would be recovered from a similar screen in diploids. Nevertheless, the conventional TILLING approaches are tedious and time consuming (Till et al. 2006). A total of 2735 tetraploid Kronos and hexaploid Cadenza EMS-induced mutants were sequenced for exome-captured DNA using NGS technology (Krasileva et al. 2017). More recently, genome-editing technologies provided additional tools to generate DNA variation in polyploid wheat (Shan et al. 2014).

Zong et al. (2017) reported complete editing of all the three homoeologs of target genes. Further optimization would ensure an efficient genome editing system for wheat, with more versatile applications such as targeted gene insertion or replacement of an allele (Zhao et al. 2016). CRISPR/Cas9 system with microspore technology can be exploited to induce genetic modifications in the wheat genome (Bhowmik et al. 2018).

3.5 Comparative Genomics

A consensus map of 12 grass family genomes including wheat is available for cereals, that represents chromosome segments of each genome relative to rice on the basis of mapping of anchor DNA markers (Devos and Gale 2000). Comparative genomics in wheat cover study of evolution and isolation or characterization of genes utilizing the rice genome. Comparative genomics approach has been used to examine several genes, like genes for glume coloration and pubescence (*Bg, Rg*), gene for grain hardness, pairing genes, *Ph1*, gene(s) controlling preharvest

sprouting (PHS), receptor-like kinase loci, and the *Pm3* gene, that confers resistance against powdery mildew (Huo et al. 2006; Gupta et al. 2008).

Earliest reports of colinearity using molecular markers in cereals, was amongst wheat A, B, and D subgenomes (Devos et al. 1992), followed by the high-gene density regions of wheat and barley. A gene density of one gene per 4–5 kb at the *Lrk10* locus was observed in wheat and its orthologous region in barley, exhibited similarly with *A. thaliana* (Feuillet and Keller, 2002). The leaf rust resistance gene locus *Lr10* cloning was made feasible in bread wheat through chromosome walking utilizing conservation of colinearity between homoeologous A genomes of the hexaploid wheat and diploid einkorn wheat (Stein et al. 2000). Except in intergenic regions, *Lr10* locus along with LMW/HMW loci of diploid wheat, on comparison with their orthologs from tetraploid and hexaploid wheats, were found to be highly conserved. On the basis of divergence of intergenic DNA (transposable elements) the Tetraploid and hexaploid wheats were predicted to have diverged about 800,000 years ago. The diploid from the tetraploid/hexaploid lineage divergence was projected to have transpired about 2.6–3 million years ago (Isidore et al. 2005). Despite initial demonstration of colinearity using molecular markers, several reports suggested interference of microcolinearity in many regions thus complicating the use of rice as a model for cross-species transfer of information.

In silico studies by Guyot et al. (2004) confirmed mosaic conservation of genes within a novel colinear region in wheat 1AS chromosome and rice 5S chromosome. Similarly numerous chromosomal rearrangements were determined on comparison of 4485 physically mapped wheat ESTs with rice genome sequence data belonging to 2251 BAC/PAC clones (Sorrells et al. 2003). Sequence analysis of the long arm of rice chromosome 11 for rice wheat synteny also supported these findings (Singh et al. 2004).

The grass genus *Brachypodium* has recently emerged as a superior model system for wheat of genus *Triticum*, since the divergence of the two genera (35–40 million years) is more recent as compared to wheat and rice divergence (Hasterok et al. 2006). Additionally, sequencing of *Brachypodium* in the coming future will further help in the in depth analyses of colinearity or synteny among the grass genomes. On comparison of *B. sylvaticum,* 371 kb sequence with ortholog regions from wheat and rice, wheat and *Brachypodium* demonstrated perfect macrocolinearity, while rice depicted ~220 kb inversion comparative to *Brachypodium* sequence. Furthermore, additional orthologous genes were recognized in *Ph1* region, among the related species wheat and *B. sylvaticum* than between wheat and rice, thus once again signifying the relative utility of *Brachypodium* genome as a superior substitute than rice for wheat comparative studies (Huo et al. 2006).

3.6 Dynamic Wheat Transcriptomes

Decoding wheat genomic sequence is the first step in order to understand its physical structure and composition, which also provides genomic infrastructure for mapping of actively transcribed regions, especially during the plant development

process and adaptation to biotic and abiotic stresses. To identify expression patterns of all the genes and their products in the wheat genome transcriptional profiling is done. This information facilitates thorough examination of gene function and regulation, as well as their crosstalk networks in the complex biological processes. cDNA microarrays were the main tool of wheat transcriptome study since long (Wan et al. 2008). Although, expression information from microarrays is not complete because of the predetermined number of probes on them, which in turn mainly relies on the genomic annotation quality. Expression patterns are envoy for cDNA arrays, indirect determination of gene expression levels by hybridization signals are hurdled by inaccuracy in quantifying real transcript numbers in definite cells or tissue (Wang et al. 2009).

Utilizing NGS technologies (RNA-seq) for sequencing RNA pools allows a systematic analysis of the complete transcriptional site, shaping genome-wide gene action and quantitative alternative splicing (Feng et al. 2017). A cell specific transcriptome profiling of homoeologous genes in the developing wheat grain determined distinctive co-expression clusters. These analyses reflect the spatio-temporal development of gene behavior during the budding endosperm of wheat (Pfeifer et al. 2014). No universal genome governance, as expected prior to this work, was found, but there was cell and stage-dependent genome dominance. The work provides exceptional information about subgenomic interactions and its outcome on gene transcription in different cell type in wheat grains (Pfeifer et al. 2014). These analyses determine the expression patterns of the whole wheat gene model set and the link between gene function, expression, and its regulation. The profiling of four stages of early wheat reproductive developments i.e. from spikelet initiation to floral organ development, have been studied and the results provided the primary information of gene regulatory clusters in inflorescence growth in wheat and identified prospective targets for wheat yield enrichment (Feng et al. 2017). Recently, Dalal et al. (2018) identified 2783 differentially expressed genes (DEGs) in wheat heat tolerant genotypes Raj3765 and 2638 DGEs in drought susceptible genotypes HD2329. Several genes related to cell wall biosynthesis and ROS metabolism were significantly upregulated in Raj3765.

3.7 NGS-Based Genotyping Divulges the Genetic Diversity of Wheat

The most proficient SNP (single nucleotide polymorphism) detection approach utilizes sequencing mRNA pools since it is relatively simple and economic (Cavanagh et al. 2013). Transcript assemblies can be employed to generate probes for specific resequencing of low-copy genomic regions (Jordan et al. 2015). The capturing of exome can also be used to target specific genes classes (Wulff and Moscou 2014). An expanded polymorphism discovery was executed by re-sequencing 62 different wheat lines utilizing whole exon capture (WEC) generated from an earlier genome assembly of Chinese Spring (Jordan et al. 2015). To determine the genetic diversity of the major global wheat growing areas, wheat lines including cultivars and

landraces were included, for obtaining a better understanding to profile the wheat current genetic diversity.

Further, the evidence supported the role of gene presence, absence variation as the cause of variation in agronomic traits. Genetic diversity across 18 wheat cultivars was explored by the generation of an improved Chinese Spring reference genome sequence (Montenegro et al. 2017). A pangenome of 140,500 ± 102 genes was predicted, with a core set of 81,070 ± 1631 genes and an average of 128,656 genes in each cultivar. In addition to variation in gene Role of gene presence, absence and variation cannot be ruled out as the cause of variation in agronomic traits. Genetic differences across 18 wheat cultivars was explored by the generation of an improved Chinese Spring reference genome (Montenegro et al. 2017). A pangenome of 140,500 ± 102 genes was analyzed, with a core set of 81,070 ± 1631 genes and an average of 128,656 genes in each cultivar. In addition to the gene presence or absence, more than 36 million inter-varietal, SNP (single nucleotide polymorphisms) were identified. A cost-effective approach to break down the genetic diversity of wheat accessions is genotyping-by-sequencing (GBS), which is a restriction enzyme-based approach that sequence only the short regions close to the restriction sites consequently representing a minute fraction of the complete genome. This technique does not depend on a predetermined set of SNPs, so it can discover population-specific variants.

The equitable nature of GBS makes it valuable for breeders (Poland et al. 2012), regardless of the complexity in downstream Bioinformatics compared with those based on array-based technologies. Nevertheless, NGS-based techniques have provided a infinite amount of genetic information for wheat and have largely contributed to wheat breeding by giving information that was earlier nearly impossible to obtain in wheat. For example, high-resolution GWAS (genome-wide association studies) has now developed into a custom tool.

3.8 Identification of QTLs for Agronomic Traits

Elite germplasm resources with high yield and quality potentials is of utmost importance for development of wheat cultivars. Therefore looking for such resources has been a continuous process for breeder community. Around ~308 QTLs/marker-trait associations (MTAs) have been reported for agronomic traits (Gupta et al. 2017). However, only few QTLs were major QTLs (explaining ~20% phenotypic variation); some of these were found to be stable QTLs and the other QTLs unstable. There are nine major and stable QTLs for agronomic traits explained ~20% to ~45% phenotypic variation (PV). Four of these QTLs were identified for grain yield, of which two QTLs were mapped on chromosome 4A. One QTL each were located on chromosomes 3B. Two stable QTLs each for 1000 grain weight and days to heading.

Remaining one stable QTL identified for days to maturity. It is very essential to understand the genetics of heading date and other agronomic traits to develop elite cultivar that can withstand global climatic changes. Yu et al. (2014) identified a total

of 32 QTLs on 13 chromosomes associated with agronomic traits such as days to heading (6 QTLs), KW (3 QTLs), spike length (6 QTLs), SN (6), TKW (04) in *Agilopis tauschii* ssp. *tauschii.* Derived synthetic wheat.

Under field conditions, wheat crops experience a combination of abiotic stresses, however many studies on the geneic regions/QTLs identification for traits under single stress field conditions were carried out. Conversely, QTLs associated with agro-morphological traits under heat stress and drought conditions at terminal stage in wheat were recently identified (Tahmasebi et al. 2017). The most important QTLs for thousand grain weight (TGW) were identified on chromosomes 1D, days to heading (DH) on chromosome 1B followed by yield related traits on chromosome 7D. Further study showed that common QTLs for either yield related, TGW and DH on 7D were validated in heat and drought trials. Bhusal et al. (2017) identified 24 QTLs associated with heat tolerance in wheat tested under timely sown (normal) and late sown (heat stress) conditions. Maximum number of QTLs were identified in chromosome 2A followed by 6D and 2B. The QTLs were detected for six QTLs for grain weight and grain numbers (6), four QTLs for grain filling rate, three for TGW and grain yield followed by two for grain filling duration. The LOD score pinpointing that QTLs varied from 3.03 (Qtgns. iiwbr-6D) to 21.01 (Qhsitgw.iiwbr-2A) explaining ~11.2% and 30.6% phenotypic variance.

3.9 Genes Involved in Abiotic Stress Tolerance

A study on meta-analysis of wheat QTLs associated with adaptation to heat and drought stress lead to identification of 502 QTLs associated with drought stress, 234 under heat stress. Only 19 MQTLs responsive for drought tolerance distributed across 13 chromosomes and 43 Meta-QTL (MQTL) regions that were co-localized for both heat and drought were reported. Acuna-Galindo et al. (2015) reported candidate genes underlying the five MQTLs and these MQTLs are mainly involved in stress signaling, antioxidative activity, and storage protein; around few of these candidate genes also involved in vesicular traffic regulation. Besides GWAS (genome-wide association mapping) approach involving ERA1-B and ERA1-D response to enhanced abscisic acid (ABA), 1-FEH-A and 1-FEH-B for fructan–exohydrolase; and DREB1A (dehydration responsive element binding) for drought tolerance in wheat have also been carried out (Edae et al. 2014). Transcription factors (TFs) also known as sequence-specific DNA binding factors is a protein that controls regulations of genes and gene clusters. A number of genes encoding TFs and involved in a two-component structure are relevant to drought tolerance (Gahlaut et al. 2014). Using genomics approach, 45 TFs mapped on 16 wheat chromosomes were habouring 56 major QTLs for 13 traits like coleoptile length, DH, TGW, CMS (cell membrane stability), chlorophyll content, DM (days to maturity), water soluble carbohydrates, stem reserve mobilization, ABA accumulation, harvest index and grain yield for drought stress.

3.10 Conclusion

The application of NGS technology has dramatically expanded our knowledge of genomics. Mainly for wheat where functional genomics would certainly not have been achievable by traditional sequencing machines. In the foreseeable future NGS would enable the pursuit for superior quality wheat reference genomes and further resequencing of a huge number of cultivars or wild relative accessions of wheat. With the availability of high quality genotyping platforms and rich genetic resources, wheat functional genomics has entered into a new phase. A comprehensive survey of genetic diversity in wheat, including landraces and wild relatives, would deepen our understanding of the genetic basis underlying domestication and evolution of this important cereal crop. The advent of the NGS technology combined with information on genetic diversity and bioinformatic pipelines would provide the thrust for wheat to catch up with other crops in studies on functional genomics thus, moving forward towards the era of genomics-assisted wheat breeding.

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Current Trends in Biotechnology: From Genome Sequence to Crop Improvement

4

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Abstract

Rapid progress has been made in the field of biotechnology since its inception from the tissue culture to now next-generation sequencing technology. The tools of biotechnology now routinely used to assist the conventional approaches of crop improvement and other field of science. Understanding the genetic make of crop species is more important for devising the strategies for the improvement. Advances in the sequencing technologies have helped in rapid discovery and genotyping of molecular markers and shifted the focus of molecular markers from the DNA fragment polymorphism to the sequence based polymorphism. The sequence information is also valuable source for identification of the function of the genes with functional genomics approaches. Over the time, a large amount of genomic information have been generated which can be good source for understanding the biological phenomenon by developing the simulation models. This has opened era of systems biology wherein biological phenomenon can be studied at the systems level. This will help in understanding and translating the genomic information for devising the strategies for the crop improvement. Here we have discussed the advances made in the structural and functional genomics and how this information can be integrated to understand the biological mechanism at system levels. We have also discussed the advances made in the molecular breeding and the transgenic approaches for crop improvement. At the end we have discussed the concern over the genetically modified crops and possibilities to develop the next generation crops using genomics approaches.

Keywords

Structural genomics · Functional genomics · Omics · Marker · Transgenic

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4.1 Structural Genomics

Structural genomics aims to characterize the genome structure in terms of nucleotide sequences. Genome sequence information can provide insight into architecture, evolution and novel aspects of crop genomes. Plant genomes vary greatly in size and complexity and they are having dominant features like whole genome duplication and large number of repetitive elements (Bennett and Leitch 2011). It has been observed that even low quality reference genomes can be the potential source for crop improvement through molecular breeding. Till date, 93 plant genomes have been assembled and annotated and are available in public domain (https://phytozome.jgi.doe.gov/pz/portal.html). Moreover, the number is increasing with the advances in the sequencing technologies and reduction in the cost. With the availability of a variety of sequencing technologies it is now possible to use specific technologies for different applications in crop improvement. For instance, long read sequencing technology can be utilized for the sequencing of the core and pan genomes as it provides the easy and accurate assembly. Haplotypes and structural variation can be better accessed through the linked-read and whole-genome sequencing. Similarly, exome sequencing and genotyping-by-sequencing (GBS) can be utilized for the allele mining and single nucleotide polymorphism (SNP) discovery (Bevan et al. 2017). The advance in the sequencing technology and assembly is discussed in more details with more emphasis on the long reads sequencing and recently introduced Oxford Nanopore Technologies.

4.1.1 Advances in the Sequencing Technology: Era of Long Reads

Since the methodology of the gene sequencing was discovered by the Sanger and others in 1975 (Sanger and Coulson 1975; Sanger et al. 1977), sequencing technology continues to advance with improvements in read length, accuracy, and throughput, opening new applications in a broad range of species (Fig. 4.1). The first-generation sequencing technology was based on the Sanger dideoxynucleotide method and three major platforms namely Applied Biosystems (ABI) capillary-based sequencer, Megabase and Beckman Coulter were available. These platforms were not suitable for large genomes because of high cost and low throughput. During 2005, era of second-generation sequencing (SGS) platform begun with the introduction of pyrosequencing based Roche's 454 GS20 with reduced sequencing costs and increased throughput, although with reduced read length and accuracy. Later on Illumina (Hiseq) and Applied Biosystems (SOLiD) platforms were introduced with even shorter reads and lower accuracy, but greater throughput. Over the time the sequencing cost has reduced with increased throughput but accurate assembly of the genome with shorter reads created the major challenge. The relatively shorter reads cannot fully reconstruct repetitive regions, leading to fragmented assemblies and collapsed regions demanding the requirement of new platform giving the relatively longer reads. With this need, the third generation of sequencing

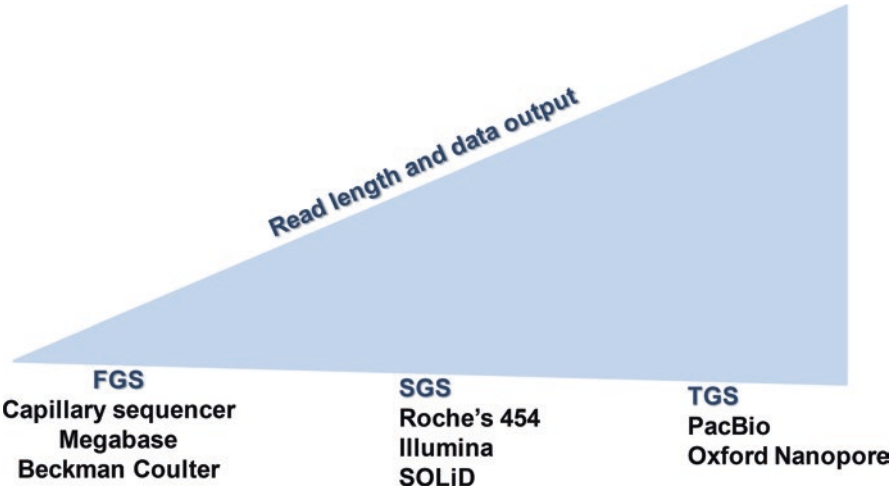

Fig. 4.1 Developments made to the sequencing technologies in terms of read length and data output. *FGS* first generation sequencing, *SGS* generation sequencing, *TGS* Third generation sequencing

(TGS) has begun with PacBio and Oxford Nanopore sequencing technology which are giving relatively longer reads. The TGS technologies, directly target single DNA molecules, and perform the real time sequencing. Apart from this, there are three important improvements in TGS platforms: (i) increase in read length from tens of bases to tens of thousands of bases per read; (ii) reduction of sequencing time from days to hours (or to minutes for real-time applications); and (iii) reduction or elimination of sequencing biases introduced by PCR amplification (Schadt et al. 2010). Apart from Helicos fluorescent sequencing (http://seqll.com/), the first successful single-molecule real time (SMRT) technology was introduced by Pacific Biosciences (PacBio, http://www.pacb.com/). With modern reagents and sequencing kits, the typical throughput of the PacBio RS II system is 0.5–1 gigabytes (GB) per SMRT cell, with a mean read length of roughly 10 kilobases (kb). Nonetheless, PacBio reads have a significantly higher error rate (10–15%) than SGS reads (>2%) (Nagarajan and Pop 2013). Fortunately these sequencing errors are randomly distributed, the rates can therefore be greatly reduced through the use of circular consensus sequencing (CCS) (Larsen et al. 2014), where a single molecule template and its complement strand are sequenced multiple times to generate a unique consensus. In 2014, Oxford Nanopore Technologies (ONT) released a new TGS platform, the MinION device, through an early access program (The MinION Access Program, MAP). The read length profile of the ONT data is very similar to that of PacBio, with a maximum length up to a few hundred thousand base pairs (Ashton et al. 2015). However, ONT reads have error rates higher than PacBio reads, with accuracy ranging 65–88% and in addition, the throughput per MinION flow cell run is not very stable at the moment, varying from below 0.1 to 1 GB of raw sequence data (Ip et al. 2015a, b). Due to its small size and low equipment cost, the MinION

sequencer is attracting considerable interest in the genomics community, particularly for pathogen surveillance and clinical diagnostic applications, as these areas would benefit from the real time nature of this sequencing platform. MinION is the portable real-time device for genome and transcriptome sequencing. The MinION generates data in real time which allows simultaneously data analysis during the experiment. Each consumable flow cell can now generate 10–20 Gb of DNA sequence data. Ultra-long read lengths are possible (hundreds of kb) as you can choose your fragment length. It weighs under 100 g and plugs into a PC or laptop using a high-speed USB 3.0 cable. No additional computing infrastructure is required. Not constrained to a laboratory environment, it has been used up a mountain, in a jungle, in the arctic and on the International Space Station. The MinION is being used for a number of biological analysis techniques including de novo sequencing, targeted sequencing, metagenomics, epigenetics and more. The advantages of the technology includes

- *Longer reads*: The technology gives the longer reads of more than 200 kb, covering the repetitive region of the genome and there by facilitating the easy assembly and phasing of the genome.
- *Real time analysis*: It enables real time sequencing, where reads are available for analysis as soon as they have passed through the sequencer. It allows the immediate access to the data and rapid insight into status of the data.
- *Easy and rapid preparation*: The required time and skill for the library preparation is not demanding and cost of the required material is also less compared with the other platforms.
- *Easy multiplexing*: It allows to run different experiments in a sequence on one flow cell or device with easy barcoding system.
- *Accessibility*: It has easy access with no capital cost, easy install and no additional lab infrastructure requirements.

4.1.2 Advances in the Bioinformatics Tool: Long Read Mapping to Optical Maps

Second-generation sequencing (SGS) has advanced the crop genome sequencing approach. However, repetitive DNA sequences in crop species often lead to incomplete or erroneous assemblies because of short reads obtained from the SGS platforms. These challenges were overcome by the introduction of long-read sequencing platforms discussed in the above section. These long reads were then used in the optical mapping to produce high-quality assemblies for complex genomes. Optical mapping is a light microscope-based technique that captures images of restriction sites to produce fingerprints of DNA sequences (Schwartz et al. 1993). Earlier the technique was used to assemble the small genome of microbes (Zhou et al. 2002, 2003, 2004) but with increase in throughput and semi-automation it was used to assemble many complex genomes (Niedringhaus et al. 2011). Optical mapping has proven to be useful for genome scaffolding and structure variation analysis, and

provides valuable physical linkage information without the reliance on recombination and genetic maps. In most studies to date, optical mapping was mainly used to validate assemblies, and few studies used optical mapping as the principal method of genome scaffolding or structural variation detection. Optical mapping competes with other physical mapping approaches, such as Hi-C and the Dovetail ChicagoTM method, and, because both continue to advance, it is unclear which is likely to dominate in the future. Long-read sequencing and optical mapping technologies have offered new solutions to accurately assemble and compare large and complex crop reference genomes. Accurate reference assemblies facilitate the identification of candidate genes for agronomic traits, information which can be applied for molecular breeding. As long-read sequencing and optical mapping technologies continue to develop and are applied for crop genomes, we will gain a greater understanding of crop genomic diversity, evolution, and gene function which will accelerate the production of improved varieties. Limitation of long reads sequencing technology like high error rates, low throughput, and high costs were overcome with recent improvements and the development of novel algorithms.

4.1.3 Marker Discovery

Apart from the understanding the genome structure, the genomic information can be used for the marker discovery, which subsequently used for molecular breeding in crop improvement. With the availability of sequencing information the focus of molecular marker has been shifted from the restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), microsatellites to SNP. Re-sequencing of the crop species allows discovery and high throughput genotyping of SNP markers as well as analysis of germplasm allelic diversity based on allele mining approach. Genome wide availability, high degree of polymorphism and multiplex analysis made it has marker of choice in many breeding programme. High-throughput array-based SNP genotyping technologies are in place which is capable of generating between a few thousand to over one million SNPs per run (e.g. Illumina BeadArray™, Affymetrix GeneChip™ technology) are available for many crop species (Barabaschi et al. 2016). Genome wide SNPs can be promising makers for mapping and quantitative trait locus (QTL) analysis. Recent development of the Infinium 8303 Potato Array has provided a genome-wide set of SNP markers that can be used in tetraploid mapping and QTL analysis (Hamilton et al. 2011). Similarly, recent advances in the development of statistical models that incorporate allele dosage information (the number of copies of each allele at a given polymorphic locus) have significantly increased the power of SNP data to detect recombination between loci and for interval mapping of QTL in autopolyploids (Hackett et al. 2013, 2014). Dense linkage maps were developed by analyzing SNP dosage in tetraploid potato. It has provided detailed information about the location of, and genetic model at, quantitative trait loci (Hackett et al. 2014).

4.1.4 From Metagenomics to Single Cell Genomics

The sequencing technologies have potential to explore the microbial discovery from the environment. Microbial discovery and diversity analysis is impeded by the inability to cultivate most microorganisms hence culture-independent approaches have been employed to study microbial discovery and identification known as metagenomic sequencing. It has gained the popularity since they appeared in 2004 (Tyson et al. 2004; Venter et al. 2004). But the meatagenomic approach is often limited by the generally fragmented nature of metagenome assemblies from short-read sequence data and by the lack of single cell resolution. This limitation has led to era of single cell genome sequencing where single cell DNA genome sequencing involves isolating a single cell, performing whole-genome-amplification (WGA), constructing sequencing libraries and then sequencing the DNA using a next-generation sequencer. Earlier the methodology of single cell genome sequencing was limited to the human where it was used to decipher genetic mosaicism for disease studies. Initially PCR and isothermal amplification -based methods were used for Genome sequencing of individual human cells (Zhang et al. 1992) and (Dean et al. 2001, 2002; Zhang et al. 2001) and further extend for bacteria and archaea (Raghunathan et al. 2005; Zhang et al. 2006). Sequencing single cell genomes of microbes will provide the discernible genetic heterogeneity within populations of closely related organisms whereas metagenome assemblies often collapse and failed to identify the strain heterogeneity. Microbial single-cell sequencing has potential role into microbial ecology, evolutionary biology, biotechnology and human health. Single-cell genomics and metagenomics approaches has widespread utility and is novel tool for addressing scientific questions (Woyke et al. 2009; Hess et al. 2011; Dupont et al. 2012; Campbell et al. 2014; Dodsworth et al. 2013; Wilson et al. 2014; Eloe-Fadrosh et al. 2016).

4.2 Functional Genomics

There are three aspects to study the function of the genome by interfering with the gene function, sequencing the gene product and identify the genetic factors that underlie complex traits. Every aspect has its own strength and limitations and development in the next-generation sequencing (NGS) technologies has fasten the process of studying the genome at functional level. The field of functional genomics attempts to describe the functions and interactions of genes and proteins by making use of genome-wide approaches, in contrast to the gene-by-gene approach of classical molecular biology techniques. It combines data derived from the various processes related to DNA sequence, gene expression, and protein function, such as coding and noncoding transcription, protein translation, protein–DNA, protein–RNA, and protein–protein interactions.

4.2.1 Interfering with Gene Functions: RNA Interference to RNA Editing (CRISPR/Cas9)

Genome editing is the process of generating the targeted modification to a DNA sequence in living cells. It can add, remove, replace, or modify existing DNA sequences, including specific chromosomal rearrangements or modification of gene expression (Germini et al. 2018). Genome editing technologies have ability to make precise changes in the genomes of eukaryotic cells. From last couple of years, programmable enzymes like zinc-finger nucleases (ZFNs) and transcription activator-like effector nuclease (TALEN) that uses protein–DNA binding were routinely used in the site specific genome editing (Gaj et al. 2013). These technologies require new protein design and validation for each experiment, restricting their wide adoption. A recent advance in this arena is programmable nucleases, based on the RNA–DNA hybridization, the CRISPR/Cas system. A major difference comparing CRISPR/Cas9 with TALENs and customized zinc finger endonucleases lies in the fact that the same endonuclease can be targeted to different genomic sequences of interest by simply designing new single-guide RNAs (sgRNAs). It has revolutionized our ability to investigate the gene function and can potentially be used to correct or introduce genetic mutations to bring out the desirable changes. The CRISPR–Cas9 system offers simplicity and efficacy in virtually all cell types making them widely used by the scientific community (Barrangou and Doudna 2016). Whatever the technique, genome editing requires the generation of double-strand breaks (DSBs) or nicks in the targeted DNA, whose subsequent repair is expected to produce the desired modifications through non-homologous end-joining (NHEJ) or homologous recombination (HR). The CRISPR–Cas9 genome-editing system exploits bacterial cell machinery to repair the genome precisely at the site of the Cas9- generated Double Stranded Break (DSB). Mutations can arise either by non-homologous end joining (NHEJ) or homology-directed repair (HDR) of DSBs (Gaj et al. 2013). NHEJ produces small insertions or deletions (indels) at the cleavage site, whereas HDR uses a native (or engineered) DNA template to replace the targeted allele with an alternative sequence by recombination. Additional DNA repair pathways such as single-strand annealing, alternative end joining, microhomology-mediated joining, mismatch and base- and nucleotide-excision repair can also produce genome edits (Bennardo et al. 2008; Lenhart et al. 2013; Gaj et al. 2013; Decottignies 2013; Sakuma et al. 2016). With its wide applicability in genome editing, variants of Cas9 were developed with the specific features like *Streptococcus pyogenes* Cas9 (SpyCas9) for use as nickases, dual nickases or FokI fusion variants (Barrangou and Doudna 2016), and other variants derived from class II CRISPR–Cas systems including Cpf1 (Zetsche et al. 2015) and C2c1 (Shmakov et al. 2015). The CRISPR–Cas systems are being used in all the aspects biology to bring out the desirable modifications to the genome (Kim et al. 2014). Apart from this in recent years the technology with deactivated Cas9 (dCas9) generated single guide RNA (sgRNA):Cas9 technology has application beyond the genome editing; when dCas9 fused to a transcriptional regulators (e.g. KRAB, VP64) or acetyltransferases and demethylases it can regulate gene expression or epigenetic modifications

respectively (Gilbert et al. 2013, 2014; Qi et al. 2013). Fusing the fluorophore to the dCas9 has also made possible for sequence-specific visualization of DNA and dynamic imaging of chromatin. Manipulation of several pathways at once in the same cell is possible with the development of orthogonal sgRNA: Cas9 systems. The potato is the ideal candidate for the genome editing as gene transformation in potato is efficient, and the genome sequence of double-haploid DM and diploid RH is available (The Potato Genome Sequencing Consortium 2011). The suitability of the technique is proved by generating the stable mutations in *StIAA* (Wang et al. 2015) and *ACETOLACTATE SYNTHASE1* (*StALS1*) genes in the potato diploid and tetraploid potato using *Agrobacterium*-mediated transformation with either a conventional T-DNA or a modified geminivirus T-DNA (Butler et al. 2015). Similarly, altered starch quality with full knockout of GBSS gene function in potato was achieved using CRISPR-Cas9 technology, through transient transfection and regeneration from isolated protoplasts (Andersson et al. 2017).

4.2.2 Expression Analysis from Gene to Genome Level: RNA-seq

Global gene expression analysis (from microarray to RNA-seq) can be performed with the specific gene or set of genes or at whole genome level. Global gene expression analysis provides quantitative information about the population of RNA species in cells and tissues. It is an exceptionally powerful tool of molecular biology that is used to explore diverse aspects of cellular world (Loven et al. 2012). Global gene expression analysis uses DNA microarrays, RNA-Seq, and other methods to measure the levels of RNA species in biological systems (Schena et al. 1998; Lockhart and Winzeler 2000; Heller 2002; Geiss et al. 2008; Wang et al. 2009; Ozsolak and Milos 2011). Initial transcriptomics studies largely relied on hybridization-based microarray technologies and offered a limited ability to fully catalogue and quantify the diverse RNA molecules that are expressed from genomes over wide ranges of levels. The introduction of high-throughput NGS technologies (Schadt Metzker 2010) revolutionized transcriptomics by allowing RNA analysis through cDNA sequencing at massive scale (RNA-seq). Several developments have been made to this approach and allowed a comprehensive understanding of transcription initiation sites, identification of sense and antisense transcripts, improved detection of alternative splicing events and the detection of gene fusion transcripts. Recent advances in the RNA-seq, Amplicon sequencing (Ampli-seq) is a highly targeted approach that enables researchers to analyze genetic variation in specific genomic regions. The ultra-deep sequencing of PCR products (amplicons) allows efficient variant identification and characterization. This method uses oligonucleotide probes designed to target and capture regions of interest, followed by NGS. It allows efficiently discover, validate, and screen genetic variants using a highly targeted approach and supports multiplexing of hundreds to thousands of amplicons per reaction to achieve high coverage. In potato, RNA-seq has been successfully used to decipher the anthocyanin metabolism by comparative transcriptome analysis of wild and mutant type of potato tubers. Genes responsible for the biosynthesis

of flavone and flavonol and phenyl propanoid biosynthesis and translation were identified. Study also revealed the down-regulation of three key genes encoding flavonoid 30, 50-hydroxylasem, 4-coumarate-CoA ligase and phenylalanine ammonia-lyase along with up-regulation of phenylalanine ammonia- lyase coding gene at molecular levels (Liu et al. 2018).

4.2.3 Genetical Genomics: From QTLs to eQTLs

A third aspect of functional genomics is the identification of the genetic factors that underlie complex traits. It can be achieved through 'genetical genomics' where attempts were made to combine the heritability of generated omics data with phenotypic variation through genetic marker associations (Jansen and Nap 2001). The result of genetical genomics study is to identify the genomic regions that control the expression of single or multiple genes, metabolites and/or proteins (eQTLs, mQTLs and pQTLs, respectively) (Kloosterman et al. 2012). The identification of eQTL may reveal the presence of major regulatory switches controlling the expression of many genes directly or indirectly (Breitling et al. 2008). The genome wide marker data and the transcript profiles can be utilized to identify the genomic regions that are associated with observed transcript levels. The identified genomic regions are termed expression quantitative trait loci (eQTLs). These eQTLs can be a local (when eQTL is within ≤1 Mb from gene that encodes the transcript) or distal, they may also act in cis or trans depending on their location on chromosome (Civelek and Lusis 2014). Sequencing of the dihaploid potato clone (DM) in 2011 opens up a new dimension for genetical genomics experiments in potato (The Potato Genome Sequencing Consortium 2011). The nature of genetic material used and sampling strategy applied in genetical genomics studies has a high impact on the output of a genetical genomics study. Diploid backcross population is used for first genetical genomics study in terms of eQTL analysis potato. Conserved and tissue dependent eQTLs for maintaining circadian rhythms and controlling clock output genes were identified from the diploid segregating population (Kloosterman et al. 2012). It combines genomic profiling and genetics to resolve metabolic, regulatory and developmental pathways (Jansen and Nap 2001). Pooling approach of RNA profiling data were used to identify of novel candidate genes for different potato tuber quality traits (potato tuber flesh color and tuber cooking type). The identified candidate genes for tuber flesh color (*bch*) and cooking type (*tlrp*) can act as useful markers for marker assisted breeding programs.

4.2.4 Towards the Systems Biology: Understanding the Systems as Whole

Structural and functional genomics has generated huge data now there is need to translate this data into more meaningful way. With the systems biology approach, these data are used to model interactive and dynamic networks that regulate gene

expression, cell differentiation and cell cycle progression. Understanding the cells at a systems level has been facilitated by recent advancements in the genomics technologies. It has led to improved annotations pipelines of genes and their products, and genome-wide studies for understanding physiological mechanisms at molecular level. Systems biology is the solution which involves study all genes, expressed as messenger RNAs, and acting through proteins and metabolites, which play important roles in a specific cell or tissue, at a specific moment. In nutshell, the approach involves quantitative measurement of genomic information, creating mathematical models based on these quantitative data, reconstruction of how a cell reacts under different conditions, and the development of theories that will explain the large variation in different species in the way they react and respond to these conditions.

4.3 Translating the Omics Data for Crop Improvement

The genomic information on structural or functional aspects needs to be translated into the crop improvement. The genomic information can be utilized for the molecular breeding or may be translated for the development of transgenics (Fig. 4.2). Sequencing information of crop species is valuable for the marker discovery. The re-sequencing of the crop species will provide the way for discovery and genotyping of the SNPs as well as identifying the new allele in germplasm using allele mining approach. The specific gene information obtained through sequencing of the genome can be used to decipher the function using functional genomics. Gene function can be deciphered by interfering the gene function using the RNAi or newly discovered genome editing tools. The functional aspect of the genome can be studied by whole transcriptome analysis or ampli-seq for specific region of the genome. The genome wide marker data and the transcript profiles can be utilized to identify the e-QTLs. Quantitative measurements and mathematic models can be devised based on this data and it will help to understand the biological mechanism at systems level. The omics information can be translated for crop improvement using the molecular breeding and transgenic approaches. In molecular breeding, genome-wide association studies (GWAS) is often used to identify the significant relationships between the trait and underlying genetic loci. The identified QTLs are the valuable source of crop improvement and can be introgressed into the genotypes to get the desirable output. The genomic information can also be exploited through transgenic approach wherein nuclear or organelle transformation can be undertaken with specific objectives. The concern over the genetically modified (GM) crops can be overcome by the development of next generation crops.

4.3.1 Molecular Breeding

Recent advances in the genomics and phenomics technologies allow cost effective methods for the construction marker dense genetic linkage maps as well as large scale phenotyping (Van Os et al. 2006) which has facilitated GWAS (D'Hoop et al. 2008).

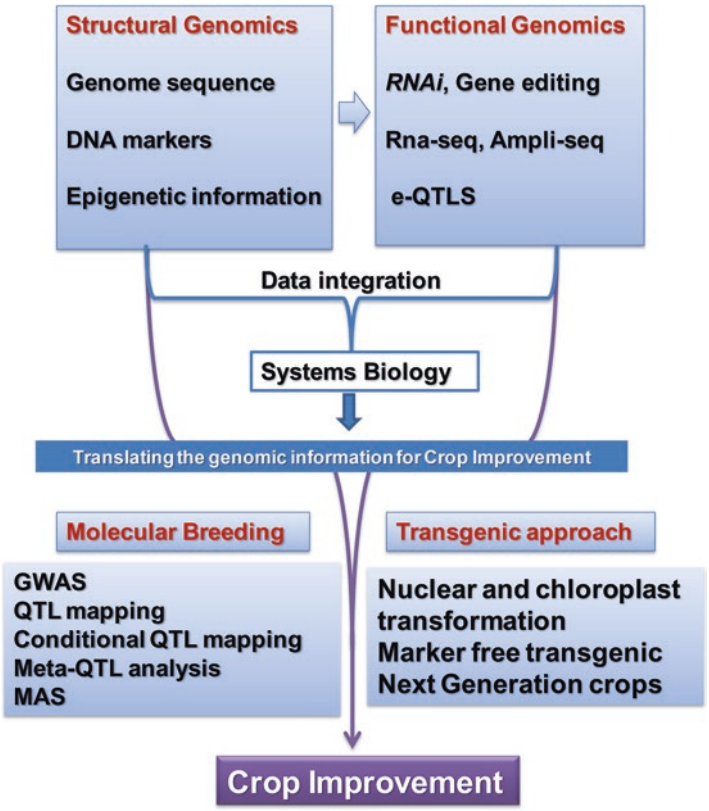

Fig. 4.2 Translating the genomic information for crop improvement

GWAS are considered alternative to bi-parental crosses for the identification of QTL because of three reasons. Association mapping using a panel of commercial cultivars increases the opportunity to detect superior alleles of relevant QTL within the breeders' gene pool. Second, GWAS has potentially a higher mapping resolution, and third it manages the diversity of the tetraploid gene pool (Nordborg and Tavare 2002; Garcia et al. 2003). The association mapping technique provides means for detecting genes underlying the variation of a trait among elite cultivars. Thus, it is complementary to linkage mapping that effectively locate genes segregating in a population originating from two individuals. Association mapping generally identifies the association of common alleles (rare alleles do not reach statistical significance), whereas a population originating from a bi-parental cross enables the identification of alleles rare in the population at large. SNPs are marker of choice in association mapping studies because of their abundance, amenability to high throughput screening, biallelic nature, reproducibility at different labs and on different platforms. They are usually co-transmitted from generation to generation as haplotype blocks that are characterized by a high degree of linkage disequilibrium (LD) and can be exploited

for gene mapping (Gabriel et al. 2002). The Illumina SolCAP potato SNP array has provided a tool for high-density genotyping tool for GWA mapping in potato. Population structure and LD were analyzed for 36 tetraploid varieties and 8 diploid breeding clones by genome-wide high-density genotyping (8303 SNPs) using the SolCAP SNP array (Stich et al. 2013). Apart from the association studies, omics approaches have boosted the crop improvement by using the novel approaches like QTL meta-analysis, conditional QTL mapping, genetical genomics, genomic selection and genotyping-by-sequencing (GBS). The conditional QTL mapping is based on the statistical model for analyzing conditional genetic effects and conditional genetic variance components. This model evaluates the conditional genetic effect of a quantitative trait at a specific developmental stage (Zhu 1995). Application of same genetic model in QTL analysis result in to the process called conditional QTL mapping. It indicates the actions of genes during development in real time (Yan et al. 1998). It is used to analyze the dynamic gene expressions associated with developmental aspects such as plant height (Yan et al. 1998) and brown plant hopper resistance (Ren et al. 2004). Conditional QTL mapping has revealed the dynamics of late blight resistance in potato and six conditional QTL were mapped for late blight resistance (Li et al. 2011). Association studies and many other conventional mapping studies have identified large amount of QTLs for a range of traits. The effect of these QTLs can be studied together by novel approach known as meta-QTL analysis where in data from multiple QTL were integrated to understand the relationships between them (Wu and Hu 2012). The general principle of a meta-analysis is to pool the results of several studies that address the same issue to improve the estimate of targeted parameters. It allows greater statistical power for QTL detection and more precise estimation of their genetic effects as it integrates the results from several studies. Hence, conclusion drawn from a meta-analysis can yield stronger results than those of individual studies and can give greater insight into the genetic architecture of complex traits (Wu and Hu 2012). Computational and statistical package like meta-QTL and Biomercator have been developed for integrating the large set of QTL data and their subsequent analysis (Veyrieras et al. 2007; Sosnowski et al. 2012). The perquisite for this approach is the availability of large amount of information pertaining to the trait under consideration. In potato late blight resistance has been thoroughly studied and mapping data for many *Rpi*-genes (resistance (R) genes to *Phytophthora infestans*) and QTL were generated and it is often observed that late blight resistance is associated with plant maturity. Based on this information the QTL meta-analysis were performed for late blight and plant maturity traits and it reveals meta-QTLs of late blight resistance and maturity place alongside on chromosomes IV, V and VIII, and overlapped on chromosomes VI and XI (Danan et al. 2011).

4.3.2 Transgenic Developments

The valuable genetic information obtained through the omics technologies and mapping studies can be used for developing the transgenic. So far genetic modification has been achieved with relatively crude but established techniques, like

Agrobacterium- mediated gene transfer and 'gene gun' that transfer the DNA from the other organisms into the cells of the target plant at random sites in the genome. The techniques can be employed to transfer large DNA constructs or multi-genes into a suitable plant host by either organelle or nuclear transformation. Organelle transformation allows transfer gene clusters or unlinked genes into a single pre-selected locus. Organelle transformation prevents pollen-mediated spread of transgenes in most species and avoids many potential unwanted epigenetic effects. The potential for the transfer of transgenes into related plant species through pollen- or seed-mediated gene flow is of concern to governmental regulators because of potential environmental and human health risks. The efforts were made in this regard by transgene bio-confinement strategies, such as male or female sterility, maternal inheritance, transgenic mitigation and excision, apomixes, cleistogamy and genome incompatibility along with the removal of the selectable marker gene using site-specific meganucleases (Liu et al. 2013). Tremendous progress have been made in the field of transgenic for various traits but implementation of this technology at farmers level is often impede by the concern over the use of genetically modified foods. The concern over the transgenic crops can be better overcome by the development of next-generation crops using high-precision editing of the plant's own genome. These approaches may reduce the need to transfer genes from other species to modify commercial crops and there by relive the public concern of GMOs. The recently developed genome editing technologies (CRISPR–Cas systems) has the potential to develop the next generation crops. In plant cells, direct delivery of CRISPR–Cas ribonucleoprotein complex can be achieved through polyethylene glycol (PEG)-mediated transfection to protoplasts, electroporation, particle bombardment, cell-penetrating peptides, or mesoporous silica nanoparticle (MSN)-mediated direct protein delivery. The plant generated from the edited cells are likely to bypass genetically modified organism (GMO) legislation as the genome editing complex is degraded in the recipient cells (Kanchiswamy et al. 2015).

4.4 Conclusion

Recent advancement in the biotechnology research has boosted the speed of crop improvement. Advances in sequencing technologies have made cost-effective sequencing and assembly of the largest and complex genome of crop species. Sequencing technologies with longer reads, single-molecule, real-time sequencing, has a major impact on accurate sequence assembly of complex genomes. With the availability of genome sequence information discovery and genotyping of the genome wide markers become feasible. These markers can be then employed in marker-assisted selection to monitor genomic loci that are linked to markers. It has also opened the new avenues in the molecular breeding likes QTL meta-analysis, conditional QTL mapping, genetical genomics, genomic selection and genotyping by sequencing which has boosted the speed of breeding at tremendous level. Great progress have been made to the functional genomics where in new approaches of interfering the gene function at precise location through CRISPR/cas9 approaches

has been devised and interestingly these has potential to overcome the concern over the use of GM crops. The transcriptome analysis using the next generation sequencing approach yielded the differential expression profiles of the large amount of genes. The functional validations of these genes have been moved one step further using the precision editing technologies. Based on the transcriptome analysis more targeted analysis is now possible with the amplicon sequencing. Despite these advances in omics technologies, the continued development of sequencing technology and computational methods is needed to improve the cost-effectiveness, quality and coverage of genome assemblies of multiple plant genomes.

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The Model Legume *Medicago truncatula*: Past, Present, and Future

5

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Abstract

Legumes are indispensable as food for us, feed for our livestock, and as a major contributor towards sustainable agricultural practices. Seed development, and root nodule symbiosis in legumes are the two main areas where majority of research is focused. Though *Arabidopsis thaliana* is a plant model with huge publicly-available resources, a model for legumes is always needed to study the unique characteristics of this family. Since last few decades, *Medicago truncatula* is being used as a model for studying plant-microbe interaction, seed development, and abiotic stress on plants. Many genomic resources have been developed, including the genome sequence, spatio-temporal gene expression data, germplasm collection, and collection of different types of mutants. This chapter describes the path followed by *Medicago truncatula* to become a model legume, along with all the above-mentioned genomic resources in detail. We have also discussed the ways of utilizing these resources in forward and reverse genetic studies. Concerted use of these resources with genome-wide analyses, molecular breeding programmes, and latest targeted genetic editing techniques has limit-less potential of empowering legumes as future food security.

Keywords

Medicago truncatula · Legumes · Model plants · Genomic resources

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5.1 Introduction

Model organisms are indispensable tools for scientific research, helping biologists since the beginning of last millennium. Unique characteristics of a model organism make it a representative of a plant family. Several characteristics are initially considered while choosing a model. First, economic importance of the plant family is the main driver behind the choice of model systems close to the crop plant. Then, intrinsic properties that accelerates laboratory handling such as small size, ease of culture, short generation time, ease of genetic manipulations such as crossing and small genome size. Two other types of properties are crucial after initial adoption of a model. Standardized and simple procedures for laboratory methods such as protein extraction, DNA and RNA isolation and transformation. The second property comprises development of public resources such as availability of reporter constructs, genetic strains, thoroughly annotated genomes, mutant collections, databases, repositories, and stock centers (Chang et al. 2016). In consequence, a well-structured and organized community of scientists will favour a rapid and efficient growth of model plant.

The Leguminosae are second only to the Gramineae in importance to humans as a source of food, feed for livestock, and raw materials for industry (Graham and Vance 2003). Legumes account for approximately one third of the world's primary crop production, human dietary protein, and processed vegetable oil. Common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), soybean (*Glycine max*), chickpea (*Cicer arietinum*), pigeon pea (*Cajanus cajan*), broad bean (*Vicia faba*), cowpea (*Vigna unguiculata*), and lentil (*Lens esculenta*) are some of the globally important economic species. Moreover, legumes produce a wide range of secondary compounds with beneficial properties, including isoflavones (Dixon and Pasinetti 2010; Dixon and Sumner 2003), which make them interesting for pharmacognosy. In 2001, 274 million tonnes of grain legumes were produced worldwide, of which 177 million tonnes were soybean (Wang et al. 2003). Legumes are also used in soil conservation, phytoremediation, lumber production, as ornamental herbs and bushes, and for extraction of gums, resins or food additives.

Legumes are the pillars of sustainable agriculture by virtue of their own supply of nitrogen by 'fixing' it (reducing N_2 to NH_3) in a symbiotic association with bacteria called rhizobia. Crop legumes and subsequent non-legume crops get nitrogen from soil. Crop legumes fix around 40–60 million tonnes of nitrogen annually, which equals to about US$20 billion of fertilizer (Graham and Vance 2003). Seeds of legumes are a rich source of dietary protein, oil, carbohydrates, fibre, minerals, vitamins, and other beneficial secondary compounds for humans and livestock. Legume seeds are second to none in protein content. This becomes more important for relatively lower socio-economic groups, since they cannot afford animal proteins. Soybean, and chickpea seeds supply a significant portion of protein in the diet of this group. Nitrogen fixation, and seed development are two major research foci in legumes.

Arabidopsis thaliana is considered as the most robust model representing the entire plant kingdom, with huge public resources available. Proposals to adopt

A. thaliana as a model plant came as early as 1940s, and the first seed stock centre was established in 1960s. First few years in Arabidopsis research were not smooth-sailing. There were only 65 publications until 1978, whereas the year 1979 saw only 7 research articles being published (Koornneef and Meinke 2010). From there, the growth in last 30 years has been remarkable. Today, roughly 4000 papers on Arabidopsis are added every year on PubMed. Nonetheless, Arabidopsis cannot be a model for research on nitrogen fixation and only a leguminous plant would serve the purpose. Moreover, though capable of nitrogen fixation, most crop legumes need additional phosphate supplement which implies a model legume to increase phosphate use efficiency in order to balance the nitrogen-phosphate ratio. Legume seed development shares many common features with Arabidopsis, but seed protein content in legumes provides an excellent opportunity to researchers to investigate the seed development in the latter. Moreover, legume seeds are excellent for their size, and overall uniformity and similarity with most of the dicot seeds. In fact, pea was the model of choice for Mendel's classic experiment. Finally, Arabidopsis cannot be a model for arbuscular mycorrhizal symbiosis, since Arabidopsis cannot be infected by the latter. Simply put, though the prowess of resources is huge in Arabidopsis, a parallel model legume has been needed.

Medicago truncatula GAERTN possesses all characteristics to be a model for legumes. First, it is an annual, self-pollinating legume producing a lot of seeds on a plant of relatively small stature, making it amenable to high-density culture (Barker et al. 1988), which grows easily in a variety of environmental conditions. Second, as a member of the Papilionoideae sub-family of the legumes, *Mt* is closely related to the majority of crop and pasture legumes, its nearest cousin in this respect being alfalfa (*Medicago sativa*), the most important forage legume in the USA (Choi et al. 2004). Third, *Mt* has a relatively small, diploid genome (haploid size approx. 550 Mbp), making it useful for both genetics and genomics (Young et al. 2005; Young and Udvardi 2009). Finally, it is relatively easy to transform, which makes it suitable for reverse genetics experiments (Chabaud et al. 2003). Since its adoption by the international community as a model species, a number of useful tools and resources have been developed for *Mt*. These include high-density genetic and physical maps for genetics research (Choi et al. 2004; Thoquet et al. 2002), various mutant populations including EMS, fast-neutron deletion, and transposon *Tnt1*-insertion mutants (Tadege et al. 2008), and tools and protocols for transcriptome, proteome, and metabolome analysis (Barnett et al. 2004; Broeckling et al. 2005; Manthey et al. 2004; Gallardo et al. 2003). An addition to the functional genomics toolkit for *Mt* is a comprehensive Gene Expression Atlas (MtGEA) (Benedito et al. 2008), which provides gene expression data for the majority of the genes in different organs and under different environmental conditions (http://mtgea.noble.org/v3/). From a draft sequence published in 2011 (Young et al. 2011), the *Mt* genome sequence has been improved to reach its fifth version providing excellent sequence and annotation datasets.

Finally, Mt. species is originated from Mediterranean region, then followed the European migration to reach offshore and be widely distributed in these areas. This distribution allowed plants to acquire adaptive mechanisms across diverse

environmental conditions, which led to the generation of a HapMap population, comprising more than 200 genotypes entirely sequenced and use in genome wide association studies (http://www.medicagohapmap.org/).

The following sections will describe some of these resources, and finally we will discuss how a better coordination of these resources will help to discover novel genes and their functions.

5.2 Pre-genomic Era

Originating around the Mediterranean Sea, *Medicago* species have spread throughout Europe. It came to the American and Australian continents much later. *Medicago sativa* (alfalfa) is used as a forage, and cover crop in both Australia and USA. Today alfalfa is also known as the queen of all the forages. With increasing agricultural importance in developed world, *Ms* attracted a lot of interest from researchers. *Mt*, being the closest relative of alfalfa, started to get attention in the latter half of last century, though at that time nobody could foresee that one day it would become one of the few representatives of all the legumes.

Today the major stock centres are The South Australian Research & Development Institute (SARDI) in Australia, the USDA National Plant Germplasm System (NPGS) in USA, the French National Institute for Agricultural Research (INRA) in France, and The Noble Research Institute in USA. Australian scientists initiated the earliest efforts to create a germplasm collection. *Mt* reached USDA collection in 1950, with a commercial strain and nine unimproved samples sent from Australia. Today, the total number of collection in these centres runs around 5000, which includes ecotypes, wild, and cultivated accessions from almost 40 countries around the world. This availability of varieties reinforces the leading position of this species as model for legumes.

Mt has seen rare reference in literature until 1970. These research articles used *truncatula* as a model for alfalfa (*Medicago sativa*), since working with alfalfa was simply too difficult. Cultivars of alfalfa are polyploids, and the entire species is strictly cross-pollinating in nature. Nonetheless, by 1990, a steady tide of articles started coming on alfalfa, thanks to the growing interest on nodule development. This led to efforts to use *Mt* as a model representative of the legume, more important fact being that it has initially became a model to understand symbiotic nitrogen fixation. This led to isolation of efficient laboratory strains (*Rhizobium meliloti*) that can nodulate and fix nitrogen in all experimental conditions (van Rensburg and Strijdom 1982). This was also the time when tissue culture protocols started to emerge (Nolan et al. 1989), some of which would later pave the path to establish the mutant collections, a major pillar of today's genomic platform. Interestingly, first few cloned genes from alfalfa were also identified around this time (Barker et al. 1988).

Barker et al. were the first to suggest that *Mt* should be developed as a model for legumes, owing to the small genome, diploid and self-fertile nature, prolific seed production potential, relatively less seed-to-seed window, and minimal genetic heterozygosity (Barker et al. 1990). With $2n = 16$ chromosomes, the genome size was

initially estimated around 500 Mbp (Barnett et al. 2004). The first *Agrobacterium rhizogenes*-mediated transformation of *Mt* was published in 1992 (Thomas et al. 1992). Several meetings amongst the *Medicago*-community were held around early 1990s in Europe and USA moving to adopt *Mt* as a model. Hitherto, Alfalfa was the system of choice, but it changed to *Mt* since then (Cook 1999).

By 1995, a shift in the model of choice was imminent, since more and more research studies used *Mt* instead of *M. sativa*. The scientific community started to put their efforts in concert to generate tools and resources for rigorous genetic and molecular studies. cDNA libraries from different tissues and conditions were prepared to clone and identify many new genes, classic example of which are *PR* genes (Szybiak-Strozycka et al. 1995; Wilson et al. 1994). Soon, expressed sequence tags (ESTs) were developed. The first EST library was a root hair enriched cDNA library (Covitz et al. 1998) consisting of 899 ESTs. Generation of random, repetitive mutant lines covering whole genome became necessary to perform high throughput forward and reverse genetic screening. EMS mutant population was first generated (Benaben et al. 1995; Penmetsa and Cook 2000) contributing to functionally characterize classical genes involved in root-nodule symbiosis (Catoira et al. 2000).

5.3 Genomic Era

5.3.1 Genome Sequencing

In parallel to cDNA and EST resources, efforts to generate genetic markers like Bacterial Artificial Chromosomes (BAC) clones (Nam et al. 1999), linkage maps were initiated. Various types of markers like CAPS, AFLPs, and RAPDs, were developed (Brummer et al. 1995; Choi et al. 2004; Thoquet et al. 2002).

With all these resources, the *Mt* community was getting ready to enter into the genomic era. The *Mt* genome project was initiated in 2002 at the University of Oklahoma, with support from the Noble Research Institute (then The Samuel Roberts Noble Foundation). Genes are mainly concentrated inside the major euchromatic regions of *Medicago* chromosomes. These regions are found in the middle of the chromosome arms. The heterochromatic regions are located at the ends of chromosome arms, and pericentromeric regions. Therefore, if BAC clones could be made from these gene-rich areas, then most of the gene spaces could be obtained by BAC-by-BAC sequencing (Young et al. 2005). The *Mt* genome was unraveled by delineating such anchored, clone-by-clone BACs (as opposed to whole-genome shotgun sequencing).

The sequencing effort of eight chromosomes was shared by different international laboratories. Among the eight chromosomes, six were sequenced in the US, funded by NSF, one (chromosome 5) was sequenced by Genoscope in France, funded by INRA, and one (chromosome 3) was sequenced in the United Kingdom with funding from the European Union and Biotechnology and Biological Sciences Research Council (BBSRC). Though the actual size of the whole genome was initially estimated to be around 500 Mbp, approximately 375 Mbp was published as

the major euchromatic region containing most of the genes. The first draft sequence of eight 'pseudomolecules' and their tentative gene annotation was published in 2011 (Young et al. 2011).

Detailed analysis of the genome supported the notion that a whole genome duplication event took place around 58 million years ago, which shaped up the present evolution of the papilionoid family of legumes. Additionally, Medicago genome has seen many gene duplications in local level. The ratio of related genes within local clusters compared to all genes in families is 0.339 in *Mt*, 1.6-fold higher than in *Arabidopsis thaliana*, and 3.1-fold higher than in *Glycine max* (Young et al. 2011).

The initial version published (v3.5) was further improved with the release of v4.0 (currently v4.2) (Tang et al. 2014). This version has seen significant improvement in both the method, and the assembly. Though a BAC-by-BAC strategy was applied for initial sequencing, the assemblies of v3.5 were complemented with Illumina whole genome sequencing in v4.0. Many sequences and genes in Mt3.5 could not be anchored to their original location in the chromosomes and were kept separate, colloquially called the 'chromosome 0'. Most of these were incorporated into the pseudomolecules of Mt4.0.This version also used actual RNAseq data to obtain better gene models, transcription start sites, and intron-exon boundaries. The presence of the RNAseq data also added confidence to the overall gene prediction. At present, the entire genome consists of 47,899 scaffolds, and 57,585 annotated genes.

5.3.1.1 Genome Resources and Browsers

Though the *Mt* genome is available through many sources in the internet, and has been integrated into many common genomic platforms, here we shall restrict the description within two major host platforms, which are the most updated and integrated ones with other resources.

The *Mt* genome database (MTGD, hosted by JCVI, USA, (http://www.medicagogenome.org/) is an integrated database hosting mainly the *Mt* genome, along with many open-sourced bioinformatics tools. These include a query-based data search engine, a genome browser (JBrowse), an integrated BLAST server, and a chromosome mapping interface. The database also contains a data mining interface, called MedicMine (http://medicmine.jcvi.org/medicmine/begin.do) where a query can generate multiple outputs like gene function, protein domain, expression analysis (RNAseq) etc. The biggest advantages of the JBrowse integrated in this resource, are the availability of multiple tracks (Fig. 5.1). Some of these tracks are not available in JBrowses elsewhere. The most important available tracks are as follows: (a) transposable elements, (b) tRNA models, (c) BACs, (d) Gaps, (e) orthologues in Arabidopsis, Glycine, and Lotus, (f) RNAseq data from nine different tissues, (g) methylome, (h) Affymetrix probe sets from Medicago Gene Expression Atlas, and finally, (i) the syntenic regions with *Lotus, Cicer, and Medicago* itself (duplication).

While MTGD is a dedicated resource for *Mt* genome, Legume Information system (LIS) is a common platform for legumes (https://legumeinfo.org/genomes). Apart from *Medicago*, LIS hosts many genomes like *Lotus*, Soybean, Common bean, Chickpea, *Arachis* etc. The *Mt* genome in LIS is connected with *Mt* genome

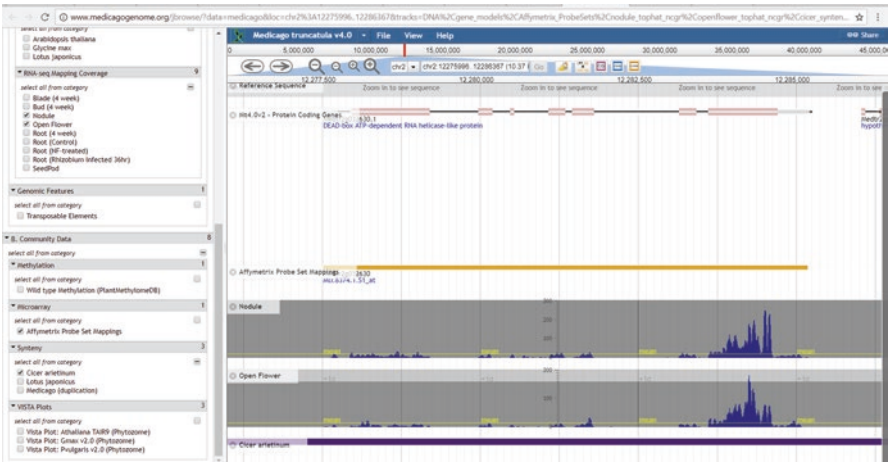

Fig. 5.1 The genome browser (JBrowse) integrated to MtGD. Different tracks being shown are (i) The gene model, (ii) The Affymetrix probe set matching to the gene (yellow), (iii) Exon-wise RNAseq data from nodule and open flower (blue), (iv) The synteny with *Cicer arietinum* (purple). (Reproduced from MtGD with permission)

at JCVI, with the physical map of 8 chromosomes, with Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Mtruncatula), and with the Medicago HapMap project (http://www.medicagohapmap.org). The genomes can also be browsed through both GBrowse and JBrowse. The browsers can connect with other resources as tracks as well. The available tracks are the gene and protein models according to both *Mt* V3.5 and *Mt* V4.0, the syntenic relationship with other legumes like *Cicer arietinum, Lotus japonicas, Glycine max* etc., the genetic markers, and the available transcripts assemblies of other organisms (data source: Dana Farber Cancer Institute, and ICRISAT).

5.3.2 Transcriptomic Resources

As in most species, identification of differentially expressed genes or creation of cDNA libraries in *Medicago* started in the 'pre-genomic era'. However, none of these efforts were targeted to cover the entire range of cDNAs. They were rather dedicated to symbiotic interactions, either with *Sinorhizobium meliloti* (Gamas et al. 1996; Wilson et al. 1994) or with *Glomus versiforme* (Liu et al. 1998). These libraries used to generate limited number of clones, probably just a subset of the total transcripts in a tissue at a given time/condition/screen. These are also called Expressed Sequence Tags (EST). The differentially expressed cDNAs were mainly identified by means of subtractive hybridization. Beginning around 2000, ESTs from different treatment and tissues started coming up (Bell et al. 2001; Covitz et al. 1998). The NSF *Mt* consortium, the Noble Research Institute (USA) and a CNRS-INRA-Genoscope collaboration in France took the responsibility of generating such

libraries as a community resource (Frugoli and Harris 2001). By 2007, the number of EST sequences submitted were more than 250,000 (Rose 2008).

Barnett et al. (2004) came up with a brilliant design of a dual-genome microarray chip in collaboration with Affymetrix. This custom designed microarray chip contained probe sets for both the partners in root nodule symbiosis, *Mt* and *Sinorhizobium meliloti*. Therefore, a change in gene expression from both the bacteria and the host could be obtained in a single chip. Affymetrix *Medicago* GeneChip became commercially available in 2005. Though designed mainly to capture the snapshots of gene expression profiles during nodule development, yet the entire *Medicago* community benefited from the chip. Soon, microarray based studies involving other organs and development conditions started using this chip. The first *Medicago* GeneChip consisted of 32,167 *Mt* ESTs and 18,733 gene predictions from *Mt* genome sequences, 1896 cDNAs from *M. sativa* and 8305 gene predictions from *Sinorhizobium meliloti*. An improved version of this chip is now available (v2.0), which allows to cover more transcripts from the main two Mt. ecotypes: A17 and R108. Indeed, the sequencing of *Mt* genome was done in A17, while most of the functional studies have been done in R108. Moreover, this chip has additional probe sets to hybridize with Alfalfa (*M. sativa*). The new chip contains enough number of probe sets to target 50,000 genes in A17, 68,000 genes in R108, and 54,000 genes in *M. sativa*.

Tiling microarray working on NimbleGen platform is another type of microarray with some added advantage. Tiling arrays have better coverage, and the results are also easier to compare on a cross-species platform. Nonetheless, the popularity of this array platform was never comparable to that of *Medicago* Affymetrix GeneChip, most probably due to the customizable nature of the former which in turn may appear as a bottleneck to many researchers. Likewise, number of studies performed using *Medicago* tiling array, for example (Verdier et al. 2013; Wipf et al. 2014), is also scarce compared to the studies that used Affymetrix GeneChip.

High throughput RNA sequencing (RNAseq) has revolutionized the way scientists consider transcriptomics as a tool. Post-2010, many researchers started utilizing this robust technique for transcriptomic experiments, for example, see (Boscari et al. 2013; Dona et al. 2013). The scopes of these studies were very focused, and were not done to build a community resource. Most of these were done in context of symbiotic relationship with rhizobia or AM fungi. In this section we would only include a few recent experiments, which covered various organs, and with publicly available data.

Vu et al. performed RNAseq to test whether parental exposure to salinity stress influences the expression of stored seed transcripts and early offspring traits in four *M. truncatula* genotypes. They detected genotype-dependent effects on the expression levels of stored seed transcripts, seed size, and germination behaviour in these genotypes (Vu et al. 2015). Roux et al. coupled two sensitive methods simultaneously to capture the snapshot of gene expression patterns in different regions of a nodule (Roux et al. 2018). The authors had dissected the different nodule zones like Fixation zone, Interzone, Zone III, etc. with the help of lasers. This was followed by RNA isolation and RNAseq analysis. The data showed that compared to roots, the nodule-enriched mRNAs comprise of zone-specific expression modules, unlike

previous notion of having a total set of nodule-expressed genes. In a similar study involving laser-capture microdissection coupled to high-depth RNA sequencing, Jardinaud et al. determined the genetic re-programming of epidermis in response to nod factors (Jardinaud et al. 2016). Zeng et al. investigated the reprogramming of secretory protein-coding genes in *Rhizophagus irregularis* in combination with three distantly-related plant species including *Medicago truncatula* (Zeng et al. 2018). Different stages of the interaction of the above-mentioned AM with *Medicago* was investigated in terms of gene expression.

5.3.2.1 Gene Expression Databases

5.3.2.1.1 *Medicago truncatula* Gene Expression Atlas (MtGEA)

Knowing the differential expression of a gene of interest in two or three different conditions is crucial in exploratory approaches. Multiple microarray experiments across several tissues and several different biological conditions help investigators to understand a global expression pattern and/or tissue specificity of a given gene. This in turn helps in forward and reverse genetics, and allows to predict gene regulatory networks, correlation of expression between two or more genes. The biggest roadblock to achieve this is the associated cost of doing so many microarray experiment together. The full potential of transcriptomic data can be exploited only when the entire scientific community will have free access to all the published data on a platform upon which they can compare between experiments. Visualization of the spatio-temporal expression of one or a group of genes is also important. The MtGEA was developed (Benedito et al. 2008) to come up with such a platform. It is a centralized resource for analysing the transcriptomic data of *Medicago*. This open-access server is hosted by the Noble Research Institute (https://mtgea.noble.org/v3). The first gene expression atlas (v1) had 18 different microarray experiments including major plant organs such as flowers, pods, leaves, petioles, stems, and also different stages of development of specialized organs like nodules and seeds. Today, the MtGEA has become an indispensable tool for the community and the number of experiments included in the server has increased up to 274, using 739 chips in total. This database covers almost every organ, along with a broad range of developmental and environmental conditions.

There are different options for searching the expression profile of a gene (or a batch of genes). This has been created for easy and user friendly data mining. Expression profiles of genes can be searched by their gene ID, the probe set ID, or by just a BLAST search if none of the above information is available. The pairwise alignment can be done against the whole intended transcript, or only the portion of the transcript covered by the probe sets. One disadvantage of BLAST search is that it may tend to give results of probe sets not intended for the gene of interest. For this reason, it is always recommended to check the alignment before drawing any conclusion. Users can also perform batch analyses like co-expression analysis, GO term enrichment, and gene regulatory network (Wang et al. 2013). The transcript viewer/the multi-transcript viewer is an excellent visual representation of the results (Fig. 5.2), where the expression level across the experiments can be viewed. This is a good representation of tissue specificity of a given gene.

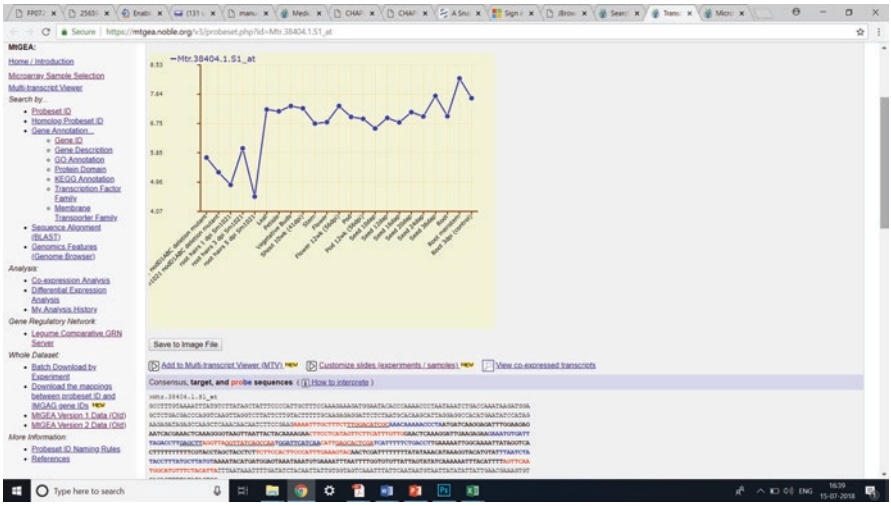

Fig. 5.2 The multi-transcript viewer from MtGEA. The sequence below is showing the whole intended target of the probe sets. The alternative red and blue colours are showing the exact sequences of the individual probes. (Reproduced from MtGEA with permission)

5.3.2.1.2 RNAseq-Based Servers

An RNAseq-based server as robust as MtGEA for hosting *Mt* gene expression data is yet to be made. This could be attributed mainly to lack of efforts to perform RNAseq experiments just in order to build a database, like it was done with Affymetrix (Benedito et al. 2008). Moreover, difficulties in cross-experiment data normalization might have resulted in the present lack of such a webserver with compiled data from different articles. Nonetheless, here we will discuss two such efforts.

Symbiomics (https://iant.toulouse.inra.fr/symbimics/) is a web server which hosts *Mt* RNAseq data from root, whole nodule, and different nodule zones dissected with laser. A user can search transcripts through keywords, sequence alignment or other source accession numbers. The output can be both relative read distribution among experimental organs being compared (%), or the read values normalized by DEseq (Anders and Huber 2010). The major drawback in this server is the relatively less number of samples included.

Plant Omics Data Centre (PODC, http://plantomics.mind.meiji.ac.jp/podc/) is a web-based resource with many genomic tools, with cross species information available. It hosts *Mt* along with many other species. At present, this server hosts 34 RNAseq samples from *Medicago*, all downloaded and curated from the NCBI SRA resource coordination database (Ohyanagi et al. 2015). The 'advanced search' function includes the option of searching the expression profiles of genes by their gene ID, keyword like Zn-finger, or by just a BLAST search. The output links include GO, orthologues, gene and protein sequence of the target, and the RNAseq-based

multi transcript viewer. The advantage of this web server over MtGEA is that the multi transcript viewer can give us a list of similarly expressing genes in the visual format, though the interpretations are similar to the co-expression network of MtGEA. This web server also provides expression data of orthologues from different species in the same window.

5.3.3 Haplotype Mapping and Genome Wide Association Studies

Small genetic variations such as single nucleotide polymorphisms or insertion/deletions are frequently inherited together. These variations which occur in similar areas of DNA generate haplotypes. Most of the time it has been seen that there is little or no genetic shuffling in between these regions, probably due to some selection pressure. Most agriculturally important traits are multi-gene traits. Availability of whole genome sequence data from different genotypes or ecotypes help in the discovery of genes involved in multi-gene traits. Today, haplotype mapping projects (HapMap) almost always follow the release of the whole genome sequence in most of the models, and *Mt* is not an exception either. The HapMap project in *Medicago* was initiated by an international consortium consisting of the University of Minnesota, the National Center for Genome Resources (NCGR), Boyce Thompson Institute (BTI), J. Craig Venter Institute (JCVI), Hamline University, INRA-Montpellier, ENSAT-Toulouse, and the Noble Research Institute. To begin with, 384 inbred lines spanning the range of *Medicago* diversity were sequenced. This provides an excellent opportunity to discover single nucleotide polymorphisms (SNPs), insertions/deletions (INDELs), and copy number variants (CNV) at high resolution among the *Medicago* lines. There were 30 deep sequenced lines (20X coverage or more), while the remainder were sequenced at 5× coverage. A dedicated web server hosts the genome sequencing data of all the lines (http://www.medicagohapmap.org/). The genome wide SNP profiles also set the platform for genome-wide-association studies (GWAS). GWAS demands population scale sampling with available whole-genome sequencing, and having enough natural variations between the samples that actually gave rise to traits. Moreover, proper phenotypic data is necessary which is used to correlate between varieties. In case of *Medicago*, availability of all the above actually helps in GWAS studies. GWAS gives a better resolution than QTL studies, thanks to the ability to dig the evolutionary recombination events between lineages (Burghardt et al. 2017).

Many GWAS studies have been published since the HapMap project has seen the light of the day. Interestingly, many of these studies were attributed to alfalfa (*M. sativa*), probably due to the direct application associated with the GWAS studies in this forage crop. Here we shall restrict ourselves in describing only a few such examples. Stanton-Geddes et al. conducted a GWAS with more than 6 million SNPs identified in 226 accessions of *Medicago*. Variations in nodulation, flowering time, trichome density, and plant height were used to identify candidate genes and/or

genetic architecture controlling the above mentioned traits (Stanton-Geddes et al. 2013). SNPs from several uncharacterized genes as well as previously-characterized genes were identified including nodulation related genes *MtnodGRP3*, *SERK2*, *MtMMPL1*, *NFP*, *MtnodGRP3A, CaML3*, and flowering time gene *MtFD*. The uncharacterized SNPs become candidates for further molecular characterization. To follow this up, Curtin et al. evaluated the real life importance of ten candidate genes identified as having naturally occurring phenotypic variation affecting nodule development in the above mentioned study. They analysed the phenotype of 17 whole-plant, and stable mutants (six Tnt1-insertional mutants, three RNAi transformants, and 8 CRISPR mutants) to evaluate the function of each of the 10 candidates (Curtin et al. 2017). Three genes (*PNO1-like*, *PHO2-like*, and *PEN3-like*) were validated by two independent mutations each, confirming the importance of those genes. The results were significant for two reasons. It confirmed the importance of novel genes, and also highlighted the power of GWAS in identifying genetic players behind multigenic traits. Le Signor et al. (2017) combined a search for genes involved in regulating protein quantity in seeds by GWAS on the abundance of 7S and 11S globulins in seeds. The genetic elements and genes carrying polymorphisms linked to globulin variations were cross-compared with pea (*Pisum sativum*). Candidate genes regulating the storage of globulins were identified in this way. Genes involved in chromatin remodelling, transcription, post-translational modifications, targeting of proteins to storage vacuoles, and transport were the highlights of the functional classes of identified genes. A co-expression network of 12 candidate transcription factors and globulin genes were analysed. The transcription factor ABA-insensitive 5 (ABI5) was identified as a highly connected hub. When a loss of function mutant of *abi5*was tested, it was indeed found to have less amount of vicilin, a 7S globulin devoid of sulphur containing amino acids. Kang et al. performed a GWA study in 220 HapMap accessions correlating with biomass and drought-related traits (Kang et al. 2015). Characterized traits included maximum leaf size, shoot biomass, stomatal density, specific leaf weight, trichome density, and few other drought-related traits. A 0.5 Mbp region on chromosome 2 correlated well with biomass. This region contains a membrane protein called PIP2 which could be a potential genetic engineering/breeding target to increase dry matter yield. Both leaf size and shoot biomass were tightly associated with a polymorphisms in disulphide isomerase-like protein sequence. An aldehyde dehydrogenase family protein, and a glutamate-cysteine ligase were two of the top genes identified by GWAS analysis of stomata density. The Arabidopsis homologs of the two above-mentioned proteins are strongly expressed in the guard cells. Together, these studies convincingly demonstrated the effectiveness of GWAS in *Mt* to identify genetic candidates to be manipulated for crop improvement.

5.3.4 Mutant Collections of *Medicago truncatula*

Standardization of an efficient transformation protocol became essential around late 1990s. Several groups published similar protocols for plant regeneration in *Mt*

tissue culture around this time (Nolan et al. 1989; Rose 2008; Thomas et al. 1992). *Agrobacterium rhizogenes*-based composite plant generation proved to be easier than *A. tumefaciens*-based transformation. Success in tissue culture techniques led to generation of mutant populations in order to facilitate forward and reverse genetics approaches of gene discovery.

5.3.4.1 EMS Mutants

Mt EMS mutants were generated as early as 1995 (Benaben et al. 1995; Penmetsa and Cook 2000) which contributed to first few classical gene discoveries involved in root-nodule symbiosis (Catoira et al. 2000). More such populations were made in the upcoming years. All these populations were made to be screened by TILLING (Targeting Induced Local Lesions in Genomes) array (Comai and Henikoff 2006). Work on this population is supported by two web servers. The REVGENUK webserver (http://revgenuk.jic.ac.uk/order.htm) is used to request a reverse screen of any candidate gene, and the phenotypic database (http://www.inra.fr/legumbase) is used to know the phenotype of a given line, or to order a line with a desired phenotype.

5.3.4.2 Fast Neutron Bombardment (FNB) Mutants

Fast neutron bombardment produces mutants which are non-transgenic in nature. The size of DNA lesions created by FNB are ideal for direct PCR detection potentially at high levels of pooling compared to conventional TILLING. Various FNB populations in *Mt* has been made (Rogers et al. 2009; Starker et al. 2006). The FNB mutation requires larger number of mutants to achieve genomic saturation, since the mutants remove either very large amount of DNA from the chromosome, or the mutation frequency becomes less when relatively smaller deletions are intended. More than 80,000 M1 FNB lines are stocked at the Noble Research Institute. Deletion-based TILLING (DeTILLING) is the method of choice for the identification of deletions in a large population (Rogers et al. 2009). A three-dimensional pooling strategy followed by PCR-based screening is employed to obtain a particular mutant. A webserver dedicated to FNB (http://bioinfo4.noble.org/mutant/) can be used to order for a screen, or to browse the phenotype of already known candidates.

5.3.4.3 Tobacco Retro-transposon *Tnt1* Based Mutants

Tnt1 fragment is a 5.3 kb long terminal repeat element from a tobacco transposon, similar to *copia* element. *Tnt1* creates a 5 bp duplication at the target site during insertion (Grandbastien et al. 1989). Southern blot and other analyses have shown the presence of 3–40 insertions, when this transposon is used to generate mutants in *Mt*.

Stability of these insertions are life-long. Most of them are genetically un-linked, and can be separated by meiotic events. These insertions showed preference for ORFs over non coding regions (d'Erfurth et al. 2003). The transposition event can be re-induced by tissue culture, which can help in the multiplication of the lines. The first success in building a *Tnt1*-based mutant in *Mt* came from the laboratory of

Pascal Ratet. Later, the Noble Research Institute took an initiative to build a genome-wide saturated mutants resource. Tadege et al. generated over 7600 independent lines representing an estimated 190,000 insertion events. (Tadege et al. 2008). Around 22,000 *Tnt1* mutant lines of *Mt* are stocked at the Noble Research Institute at present, covering around 90% of all genes (Cheng et al. 2014). *Tnt1* mutant population has been used in forward genetic screens using inverse-PCR or thermal asymmetric interlaced-PCR (TAIL-PCR) to recover the flanking sequences of the insertion (Benlloch et al. 2006; Cheng et al. 2014).

5.3.4.3.1 Medicago *Tnt1* Database

All the information regarding the *Tnt1* population such as request of mutated lines, description of mutants is available at https://medicago-mutant.noble.org/mutant/database.php. This database is a searchable database. A mutant corresponding to a gene of interest can be searched by gene ID, or BLAST search. On the other hand, known phenotypic description of all the mutants are available. Hence, users can also search for mutants of desired phenotype by description. Every output also opens a number of mutant lines carrying at least one insertion in the desired gene. In case many such lines with many insertions come out, a high and low confidence on each of the FST borders (i.e. representing mutated genes) is given. This database is also integrated with a genome browser, where the insertions are mapped on the genome. If no known line is found, the users can order for a fresh round of genetic screening to find out such mutants from the population (Table 5.1).

Table 5.1 Major genomic resources of *Medicago truncatula* discussed in this chapter

	Name of the resource	Purpose/highlight of the resource	URL
1.	*Medicago truncatula* genome database (MtGD)	Genome sequence, genome browser	http://www.medicagogenome.org/
2.	Legume information system (LIS)	Genome sequence, genome browser, phylogeny and synteny analysis	https://legumeinfo.org/
3.	*Medicago truncatula* Gene expression atlas (MtGEA)	Spatio-temporal gene expression of *Medicago truncatula,* microarray-based	https://mtgea.noble.org/v3/
4.	Plant Omics Data Center (PODC)	Spatio-temporal gene expression of *Medicago truncatula,* RNAseq-based	http://plantomics.mind.meiji.ac.jp/podc/
5.	Mutant collection at Noble Research Institute	Collection and distribution center of *Tnt1*, and FNB mutant lines	http://bioinfo4.noble.org/mutant/
6.	*Medicago* HapMap project	High and low depth whole genome sequencing of various ecotypes and wild collections	http://www.medicagohapmap.org/

5.4 Forward and Reverse Genetic Approach of Gene Function Discovery

Availability of all the above mentioned resources have revolutionized legume biology in the post-genomic era. Forward and reverse genetics have become easier with concerted use of these resources. Since *Tnt1* is the most widely-used mutant resource, we would describe the following sections with respect to *Tnt1* mutants. Nonetheless, the methods for utilizing other types of mutants are quite similar.

In forward genetic approach, users identify mutants with a desired phenotype, for example small seeds. The next step is to identify the gene where the *Tnt1* insertion has occurred. Given the multiple insertions in the same line, even after knowing the flanking sequence tags (FSTs) and identifying a candidate gene, researchers need to confirm that the phenotype of interest is really due to the disruption of the selected gene. As a next step, the users must back-cross the mutants for a few rounds and choose homozygous of the suspected gene at F2, to check whether the phenotype is persistent. For this reason, knowing all the FSTs in each line is important. The fact that *Tnt1* is inserted as a single copy at one locus is rather of advantage. With the border between the transposon and target site being intact, these latter characteristics are important for the identification of the disrupted loci (Tadege et al. 2008). The FSTs from *Tnt1* lines can be obtained using thermal asymmetric interlaced-PCR (TAIL-PCR) followed by TA cloning, plasmid isolation, and traditional Sanger sequencing. About 33,000 flanking sequence tags (FSTs) from 2600 *Tnt1* lines were identified using TAIL-PCR at the Noble Research Institute. Cheng et al. developed a novel two-dimensional DNA pooling strategy coupled with next-generation sequencing. This strategy has accelerated the process of FST sequencing by-passing the painstaking cloning and Sanger sequencing. They have produced about 380,000 FSTs from all 21,700 lines within a few years (Cheng et al. 2017). Nonetheless, some FSTs in most of the lines cannot be identified by any method combined with TAIL PCR, because of the PCR bias towards amplifying smaller fragments. To meet this shortcoming, Veerappan et al. used Illumina sequencing to obtain the whole genome of two *Tnt1* lines, and found many more FSTs compared to TAIL PCR (Veerappan et al. 2016). Use of such a method in forward genetic screens has been recently improved by the introduction of ITIS, an algorithm which retrieves *Tnt1* insertion sites of a mutant line with available low coverage genome sequencing data (Jiang et al. 2015).

In reverse genetic approach, a gene of interest is first chosen. This choice can be based upon the spatio-temporal or tissue specific expression pattern of the gene. This can be obtained from MtGEA, or PODC (see above). A gene regulatory network may also be helpful in this (Wang et al. 2013). The next step is to find a mutant having insertion in the gene of interest. Once an insertional mutant line is identified, scientists check whether the plant has any phenotype in the desired tissues. Again, given the multi-insertion nature of *Tnt1*, it is always recommended to have more than one line with one common insertion in the same gene, and checking whether

they have similar phenotype. After this, these mutants should be back-crossed with the wild type to check the persistence of the phenotype.

5.5 Future Directions, Potential, and Comparison with Other Model Legumes

With all the resources in hand, *Mt* is quickly running to become 'the Arabidopsis of legumes'. While there are other models of legumes such as Soybean, and *Lotus*, *Medicago* has gained significant boom in the race for being the best representative of legumes. While *Mt* has some advantages over others, one must not forget that there cannot be a sole representative or lone model for legumes. First, we must understand the reasons of doing research on legumes. Legumes can host *Rhizobia* as nitrogen fixing symbiotic partner. This virtue has been acquired by legumes recently in an evolutionary time scale. Further local genomic rearrangements in legumes have given rise to various types of features of nodule development. There are determinant nodules, indeterminate nodules, nodules with infection threads, and nodules where bacteria enter through cracks. Symbiosomes also vary in their shape, size, and nitrogen fixation efficiency. To understand nodule development from an evolutionary perspective, it is important to study different types of nodules. Therefore, studying different legumes and having more than one model is important. Though adopted around same time as models, the interest in *Mt* as a model has been increased significantly in comparison to *Lotus*, most probably due to better effectiveness of *Tnt1* mutant population over the *LORE1* mutant population of *Lotus*. This is evident from the number of published literature in last few years (Fig. 5.3, compare between *Lj and Mt*). Lotus was initially promoted by various Japanese and other groups of scientists, while *Mt* was promoted by the European and American consortium of scientists. Another advantage of *Medicago* is its phylogenetic relation with economically important forage crop alfalfa (*M. sativa*). In this regard though, soybean (*Glycine max)* is a model that itself is the most economically-important legume. Nonetheless, soybean suffers from the biggest possible bottleneck in the progress of a model plant: it is recalcitrant to transformation. Though Soybean has both FNB and EMS lines, lack of an insertional mutant collection makes it difficult to work with. Only recently (Sandhu et al. 2017), endogenous transposon *Tgm9* has been shown to have the potential to be used to generate a mutant population. As we have seen in case of *Mt*, for Soybean this will take several years to get a mutant population with decent genome saturation. While adopted as model around late 1990s, *Mt* did not feature in even a single research article before 1990, and the number was just 19 by 1995. An overwhelming number of more than 2100 papers have been published in the new millennium with the genomic resources being available (Fig. 5.3, source NCBI-PubMed). The trend clearly shows that more publications are on the way.

Though *Mt* is a decent model legume, it has room for improvement in future. The genome is being updated continuously with another updated version on the way. Though MtGEA is an effective platform for analysing gene expression, a similar

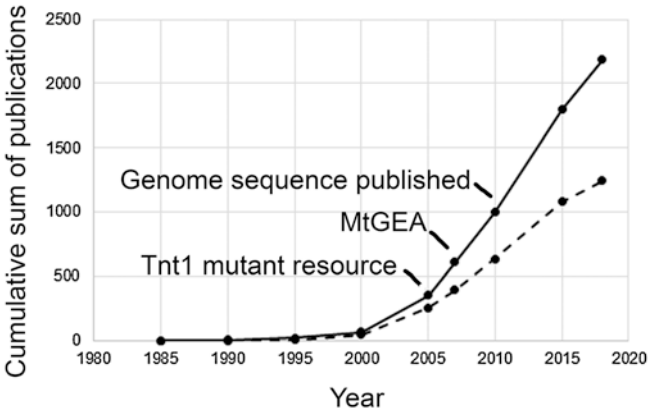

Fig. 5.3 The year-wise cumulative sum of publications of research articles in *Medicago truncatula* (solid line), and *Lotus japonicas* (dotted line). Major milestones of genomic resource development in *Medicago truncatula* are shown. (Source: NCBI PubMed)

platform of all the RNAseq data is lacking. At present, neither of the RNAseq-based servers are as effective and user friendly as the MtGEA. Few recent studies have proved that CRISPR-Cas9-based genome editing is possible in *Mt* (Cermak et al. 2017; Meng et al. 2017). More research on this area, and finally a pipeline for generating targeted point mutants in *Mt* will be an excellent platform for reverse genetics, since multiple insertions of *Tnt1* is a perpetual problem. Alfalfa, being an important forage in the Southern Great Plains of USA, have been suffering from severe drought since last decade. Genomic tools in *Mt* give an excellent opportunity to work on this aspect. Research in this area has already been started and we expect more knowledge to come up in future. Finally, nitrogen fixation in legumes is heavily affected by a lack of phosphate in soils. Legumes which can thrive in low phosphate soil is a dream for plant biologists. We hope the next decade will be crucial for the research in this frontier. Apart from alfalfa, *Mt* is also phylogenetically close to world's second largest crop legume chickpea. While both seed development and nodule development in chickpea and *Mt* share similar features, there is room for improvement in chickpea. The efficiency of nitrogen fixation is much less in chickpea compared to its closest model *Mt*. Similarly, the protein content in chickpea seeds is much less than *Mt*. Comparative genomic studies between *Mt* and chickpea can provide valuable information to understand the differences. While few efforts in this approach have been initiated, the final validation must involve *Mt* Tnt1 mutant analysis, since any such resource is absent in chickpea. Overall, *Medicago truncatula* can be considered as a Swiss knife to dissect the recent problems in leguminous crop production under changing environmental and soil-health conditions.

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Genomics and Molecular Mechanisms of Plant's Response to Abiotic and Biotic Stresses

6

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Abstract

Previous two to three decades have witnessed Abiotic (temperature, light, water, salt etc.) and Biotic (bacteria, fungi, viruses etc.) stresses in crop plants to be increasing and documented as a severe menace to global food security, making it hard for the plants to endure in such circumstances. With the fast-growing population, it is now mandatory to pace with the yield and productivity accordingly, thus protection of crop plants from the abiotic and biotic stresses is a priority. The expansion of stress-tolerant crops will be significantly profitable for the poor farmers in regions of the globe that are affected by such stresses. Similarly, a number of transcription factors/regulators play crucial roles in plant stress responses. This chapter emphasizes on the genes involved in plant's response to abiotic and biotic stresses with their molecular mechanisms to summarize the current knowledge and a step further for their better understanding. Such genes need to be properly utilized to generate resistant crop plants for future generations.

Keywords

Plant stresses · Genes · Mechanisms · Stress combination · Nanotechnology

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6.1 Introduction

Plant response to biotic stresses of pathogens, that can be described as the power of microorganisms infecting a particular host resulting in a disease (Casadevall and Pirofski 1999). Further pathogenicity is a continuously evolving personality of microorganism's in turn denting the host (Falkow 1990; Janda and Abbott 2010). The conclusion of aggressive meet among pathogenic microbial species and their hosts is based on the virulence factors exhibited by pathogens, the survival of resultant host targets, and host reaction to these factors (Mahajan-Miklos et al. 1999).A number of factors governing pathogenicity have been recognized in fungi (Sudheep et al. 2017), ranges from small molecule suppressors and host selective toxins (HSTs) (Collmer et al. 2002; Bender et al. 1999). Bacterial pathogens constitute toxins and type III effectors (Wolpert et al. 2002; Shiraishi et al. 1997). Finally, viral species delivering biotic stress as pathogenicity factors (Nehra et al. 2018; Marwal et al. 2016; Prajapat et al. 2011a, 2014) are the suppressors of post-transcriptional gene silencing (Moissiard and Voinnet 2004; Rahme et al. 1995; White and Gardea-Torresdey 2018).

Similarly, plant response to abiotic stresses is also complex and involves a number of genes and their biochemical molecular mechanisms (Shinozaki and Shinozaki. 2000; Ludwig et al. 2004; Zhu 2002; Bray 2004). Microarray studies conducted on two different potato clones revealed that a total of 1713 genes were expressing differentially, out of this 186 were found to be upregulated during drought stress. The genes were related to membrane modifications, detoxification of oxygen radicals, osmotic adjustment, protein stabilization, cell rescue mechanisms, carbohydrate metabolism etc. (Schafleitner et al. 2007). When *Arabidopsis thalina* plant was subjected to dehydration stress, it was confirmed that 6579 genes were participating into increasing or decreasing the gene products. When the plant was re-subjected to stress, a total of 1963 genes produced significantly different amounts of transcripts than the previous stress applied (Ding et al. 2013), and such genes can be detected with the help of various molecular markers as described previously (Verma and Singh 2013).The current manuscript highlights and describes our current knowledge on the genes involved in plants' response to abiotic and biotic stresses with their molecular mechanisms. In addition, this chapter records the relevance of different stress combinations on crop species and recent developments in the field of nanotechnology to help plants to tackle stresses.

6.2 Genes and Molecular Mechanisms Governing Abiotic Stress

There are genes which encode proteins such as detoxification enzymes, osmoprotectants, free-radical scavengers, heat shock proteins (HSPs) or chaperones, antifreeze proteins, LEA proteins, ion transporters, aquaporins, transcriptional factors, SOS kinase, Mitogen-activated protein kinase (MAPK), phospholipases, Calcium-dependent protein kinase (CDPK), thus all the above mentioned products help

protect the plant from various stresses (Bray et al. 2000; Blumwald 2000; Shinozaki and Shinozaki 2006; Frank 2000; Wang et al. 2003; Zhu 2001). LEA proteins are encoded by *Lea* gene during late embryogenesis. LEA proteins have chaperone-like function, which are hydrophilic in nature and function to preserve other proteins and even membranes (Chandra et al. 2004; Rizhsky et al. 2002).

During cold stress Transcription Factors such as DRE/CRT bind to DREB1/CTR-binding factor (CBF) and DREB2. The expression studies of DREB1 genes in response to abiotic stress have been thoroughly explored in a number of plant species. Further it has been proved that AtDREB1 gene expression is induced by cold and not by dehydration or high salt stress. Likewise, CBF genes reveal good expression in cold stress (Liu and Zhu 1998; Liu 2000; Medina et al. 1999; Shinwari et al. 1998). A reprogramming of the genetic machinery occurs when any plant is subjected to biotic or abiotic stress, common things gets activated are jasmonic acid (JA), ethylene (ET), reactive oxygen species (ROS), salicylic acid (SA), kinase cascades, abscisic acid (ABA) etc. result in providing sufficient defence and protection to plant (Laloi et al. 2004; Fraire-Velázquez et al. 2011; Spoel and Dong 2008; Fujita et al. 2006; El-Ramady et al. 2018) (Fig. 6.1).

Response regulator (RR) protein—plays an important role in response to plant stress. The Arabidopsis RRs are classified into two distinct subgroups, type A RRs and type B RRs. Type B RRs, include 11 members and the type A RRs, include ten members. There are at least 14 histidine kinase genes, 15 type A RR genes, and seven type B RR genes identified in rice genome. Such RR helps in perception and integration of various extracellular and intracellular signals. From 10 genes, *OsCBL2* was found to be expressing i.e. upregulated by gibberellic acid (GA). *OsCBL2* targets the aleurone tonoplast and promotes vacuolation in aleurone cells through the GA-signaling pathway. Aleurone is a protein found in protein granules

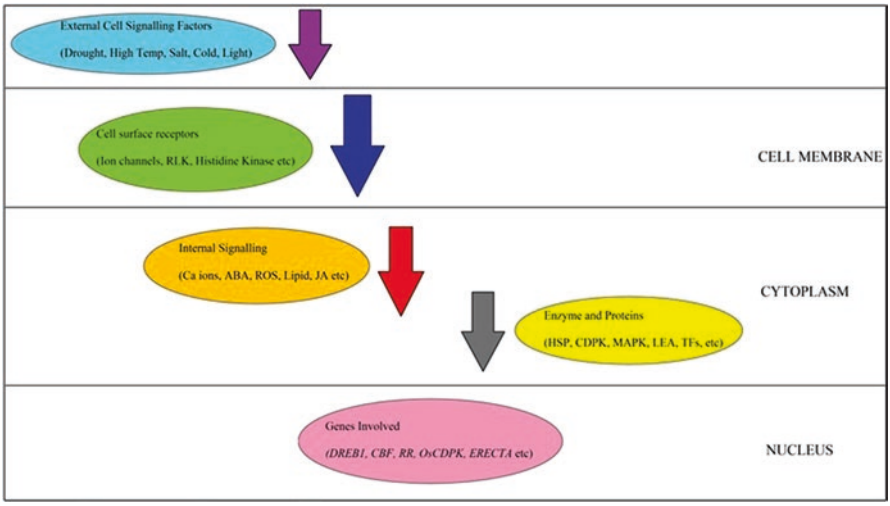

Fig. 6.1 Series of factors/signalling pathways governing abiotic stress in plants

of maturing seeds and tubers (Pareek et al. 2006; Kiba et al. 2003; Doi et al. 2004; Hwang and Sheen 2001; Besant et al. 2003; Du et al. 2007; Hwang 2005).

A number of governing pathways have been found playing a crucial role in plant stress signalling, such as MAPK (Mitogen Activated Protein Kinases), CDPK (Calcium Dependent Protein Kinase) and Calcineurin B-like protein (CBL)-CBL-interacting protein kinase (CIPK) pathways. Around 20 OsCIPK genes have been identified in relation to cold, polyethylene glycol (PEG), salinity, ABA and drought. Similarly, seven *OsCDPK* genes have been found to mount up differentially during salt, cold and desiccation. *OsMAP1* is a MAPK gene, (also called as OsMAPK2, OsMAPK5, OsBIMK1and OsMSRMK2), is known to get activated under the influence of numerous biotic and abiotic stresses, important once are low temperature, pathogen infection, salinity, ABA stress and drought. Improved tolerance to cold, salt, and drought has been widely supported by *OsCDPK7* gene product. At the transcript level, *OsCDPK13* responds to various abiotic stresses as well as to hormone levels. Seven *OsCDPK* genes have been found to accumulate differentially during cold, salt, and desiccation stress (Agarwal et al. 2002; Huang et al. 2002; Katou et al. 2007; Ray et al. 2007; Saijo et al. 2000; Song and Goodman 2002; Wen et al. 2002; Xiong and Yang 2003).

One of the biggest and varied DNA-binding transcription factors in plants is the Myb transcription factor family. The responsibility of *Myb* genes in response to abiotic stress have been deliberated in a number of plant species. Similarly, *ERECTA* gene codes for a protein kinase receptor which mediates plant responses to disease, predation and stress. Constituents of the *ASR* gene family are induced by abscisic acid (ABA), different abiotic stresses such as cold, limited light, water and salt stress. In sorghum an *AMADH* gene was identified to be associated with salt stress tolerance, osmotic stress tolerance and dehydration. In rejoinder to environmental and nutritional stresses in plants, the *AKIN* (SNF1 associated protein kinase) gene falls in the CDPK–SnRK superfamily, thus providing important regulators for amending fundamental metabolic pathways in plant response to stress (Carrari et al. 2004; Halford and Hey 2009; Joo et al. 2013; Masle et al. 2005; Petrivalský et al. 2007; Riechmann et al. 2000; Romero et al. 1998; Shpak et al. 2004; Volpe et al. 2013; Wood et al. 1996; Yang et al. 2005; Zhu et al. 2008).

6.3 Genes and Molecular Mechanisms Involved in Biotic Stress

A survey was conducted among the scientific community (plant pathologists) to identify top 10 bacterial pathogens causing pathogenicity (biotic stress) in plants, where in *Pseudomonas syringae*was considered at the top. Some of the other species include*Agrobacterium tumefaciens, Xanthomonas campestris, Erwinia amylovora, Xylellafastidiosa, Pectobacteriumcarotovorum* etc. (Rahme et al. 1997; Prajapat et al. 2013; Mansfield et al. 2012). Similarly, in the case of fungi *Magnaportheoryzae* topped the 10 list among *Puccinia spp., Fusarium graminearum, Fusarium oxysporum, Mycosphaerellagraminicola, Colletotrichum*

spp., Ustilago maydis etc. (Dean et al. 2012; Mahmood et al. 2017). Plant virologists generated the list of top 10 plant viruses in relation to biotic stress on plants being *Tobacco mosaic virus* at the first place. Other viruses in the list were *Tomato yellow leaf curl virus, Cucumber mosaic virus, Cauliflower mosaic virus, African cassava mosaic virus, Plum pox virus* and other Gemini viruses (Scholthof et al. 2011; Prajapat et al. 2011b, 2012; Sahu et al. 2014a).

Bacterial resistance coupled with hypersensitive response (HR) in plants especially necrogenic pathogens acting as bacterial pathogenicity has drawn a great deal of consideration among pathologists (Klement 1982; Goodman and Novacky 1994). A series of advancements have revealed *hrp* and *avr* genes for hypersensitive response and virulence, respectively in accordance to a handful of other genes coupled with toxin, pectic enzyme and extracellular polysaccharide (EPS) production (Alfano and Collmer 1996). Gram negative bacterial Type III effector proteins of an extracellular nature constitute type III secretion systems (TTSSs) responses when injected into the host cellular machinery. TTSSs emerging from the bacterial envelope translocate proteins into plant cell wall and plasma membrane. *Ralstonia solanacearum*, *Pseudomonas syringae, Pantoeaspp*, *Xanthomonas* and *Erwinia* are among few bacterial pathogens encompassing TTSS (Collmer et al. 2002).

Another factor contributing to pathogenic biotic stress is Quorum sensing which permits bacteria to communicate with each other by discharge and recognition of small diffusible signal molecules. Characteristics governed by Quorum sensing comprise discharge of antibiotics inhibitors, degradative enzymes, siderophores discharge, pigments discharge etc. are repeatedly requisite for pathogenesis. Bacterial species exhibiting Quorum sensing are *Pantoeastewartii, Pseudomonas aeruginosa, Ralstonia solanacearum, Pseudomonas syringae, Agrobacterium tumefaciens, Xanthomonas campestris* and *Erwinia carotovora* (von Bodman et al. 2003) (Fig. 6.2).

Fungal species causing pathogenic biotic stress are known to liberate macromolecules that postpone or thwart defence responses of the host plants making them vulnerable to a virulent microorganisms. Such compounds are called as suppressors and have been identified as glycopeptides, glycoproteins, peptides, or anionic and nonanionic glucans. Such suppressors do not exhibit noticeable damage to plant cells. Several fungal species belonging to genera Helminfhosporium and Alternaria are known to secrete such suppressors (Shiraishi et al. 1997). Toxins are a group of diverse metabolites excreted by fungal pathogens causing pathogenicity or virulence (Wolpert et al. 2002).Toxins aid in fungal infiltration into plants and facilitates in colonizing host tissues. *Cochliobolusheterostrophus* is a major plant pathogenic fungus secreting toxic metabolites resulting in undesirable effects on a range of host plants especially infecting maize plant by producing T-toxin. *Cochlioboluscarbonum* causes disease symptoms like leaf spot and ear moldin maize (Kono et al. 1981).

This T-toxin belongs to the family of linear polyketides whose chain length may spans from C35 to C45 (Levings and Siedow 1992). Maize plant reveals T toxin toxicity in the inner membrane of mitochondria of the host plant (Dewey et al. 1988). This pathogenic fungus produces a cyclic tetrapeptide functioning as a host selective toxin (He-toxin) (Walton et al. 1982; Scheffer et al. 1967). Pisatin belongs

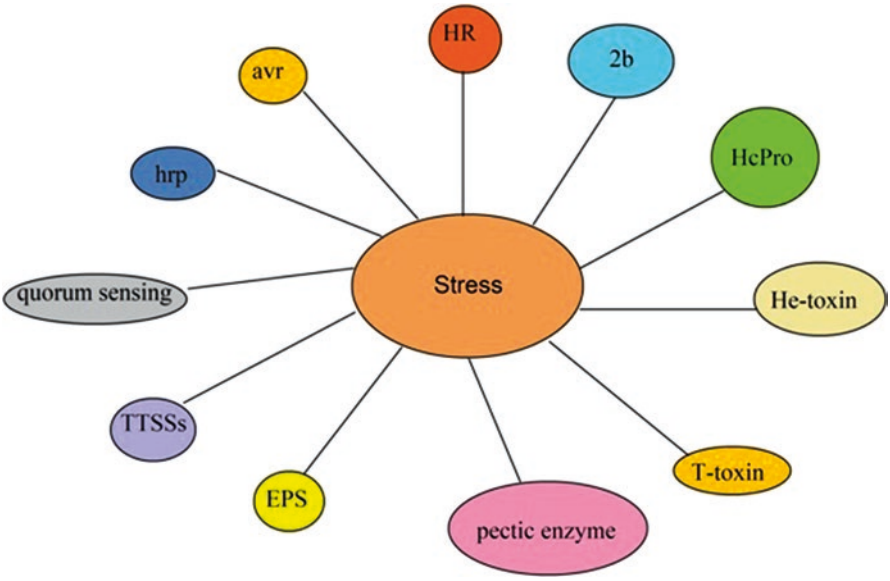

Fig. 6.2 Interacting factors governing biotic stress in plants (Add Acronyms)

to a class of isoflavonoid is a host response molecule secreted by host pea plants in response to fungal attack. To neutralize this host response, all infecting fungal species (such as *N. haematococca*) produce pisatin demethylase (pda). Fungal pda detoxifies pisatin through demethylation to a nontoxic compound (VanEtten et al. 1989; Schafer 1994).

Likewise, chloroplasts which are involved in plant photosynthesis lack RNA silencing machinery, which protect the plants from the attack of viruses and are the main centre/target of RNA virus-mediated pathogenic stress (Sahu et al. 2014b). Helper component proteinase (HcPro) of potyvirus and Triple Gene Block (TGB) potexvirus was known to suppress plant host defence mechanism via RNA silencing (Vance 1991; Gaur et al. 2012; Marwal et al. 2017; Pruss et al. 1997). Similarly, another study revealed that Cucumber mosaic virus (CMV) 2b protein also acts as a suppressor of host plant Post Transcriptional Gene Silencing (PTGS) (Ruiz et al. 1998). The breakthrough of the anti-silencing activity of HcPro and 2b offers an indirect hint that silencing suppression is possibly a shared feature of many plant viruses (Marwal et al. 2012; Moissiard and Voinnet 2004).

6.4 Combined Biotic and Abiotic Stresses Affecting Plants

A number of stress governing genes/factors have been discussed above individually, but in nature most of them occur in combination with each other interacting either independently or overlapping with plants in their mechanisms. There are

certain examples of biotic: biotic, abiotic: abiotic and biotic: abiotic stresses found naturally. Certain examples are cited below. Walnut trees are affected by biotic: biotic stress of bacterial (*Xanthomonas arboricola*) and fungal (*Colletotrichum*, *Alternaria*, *Phomopsis*, *Cladosporium* and *Fusarium* species) pathogens at the same time causing necrosis of apical meristem (Belisario et al. 2002). Grape plantations in Northern China are suffering with abiotic: abiotic stress, in which low temperature were coupled with high light intensity (Su et al. 2015). Similarly, even the Mediterranean grape vines are facing the same situation as that in Northern China (Loreto and Bongi 1989). Likewise, Barnes and Davison (1988) have studied wheat plants challenged both with low temperature and ozone stress. Ozone stress was also coupled with high salt concentration of soil affecting rice and chickpea crop fields severely (Welfare et al. 2002). Crop plants face major stress problem together, say that of weeds, heat, drought and cold; an example of combination of biotic: abiotic stresses (Valerio et al. 2013; Cordes and Bauman 1984; Patterson and Flint 1979).

The above-mentioned scenario highlights the negative effect of stress on plant growth, development, yield etc. There are some stress combinations which result in a positive manner on the plant health and proved beneficial (Table 6.1). Medicago plants was when subjected to ozone stress developed necrotic lesions and leaf chlorosis. When the same plants tested for drought stress it had wilting and even chlorosis of leaves. But when both the stresses were combined their damaging effects got nullified possibly due to low stomatal conductance. Even the reactive oxygen species (ROS) concentration showed reduction under both the stresses in combination (Iyer et al. 2013). Similarly, high CO_2 concentration proved advantageous by inhibiting the diffusion of ozone into the leaves due to reduction in the stomatal conductance (Ainsworth et al. 2008). Likewise, high CO_2 increased the biomass of lettuce when combined either with salt or high light conditions (Perez-Lopez et al. 2013).

Table 6.1 Various stress combinations having positive and negative impacts on plant health

Impact on Plant Health	Stress Combination	References
Negative	Ozone & Salinity	Mittler and Blumwald (2010)
	Ozone & Heat	Kasurinen et al. (2012)
	High Light & Drought	Giraud et al. (2008)
	High Light & Heat	Hewezi et al. (2008)
	High Light & Chilling	Haghjou et al. (2009)
	Pathogens & Drought	Prasch and Sonnewald (2013)
	Pathogens & Salinity	Xiong and Yang (2003)
	Pathogens & Heat	Zhu et al. (2010)
	Pathogens & Chilling	Szittya et al. (2003)
Positive	High CO_2& Drought	Brouder and Volenec (2008)
	High CO_2& Salinity	Perez-Lopez et al. (2013)
	High CO_2& Ozone	Ainsworth et al. (2008)
	UV & Ozone	Mittler and Blumwald (2010)
	UV & Pathogens	Bowler and Fluhr (2000)

6.5 Application of Nanotechnology

Nanotechnology is the future and a fast emerging helpful to field to protect the plants from abiotic and biotic stresses Nanoparticles are produced either conventionally or through the green synthesis/bioreductive method (Aslani et al. 2014; Das et al. 2014a; Cho and Seo 2005; Guerriero and Cai 2018). Nanotechnological applications assist in genetic modification to protect the plants from abiotic and biotic stress (Das et al. 2015; Mingfand et al. 2013). Similarly, nanosensors also may help in crop security against bacterial, fungal and viral pathogens by detecting their presence at a very early stage of infection (Ye et al. 2012; Das et al. 2014b; Busemeyer et al. 2013; Ma and Yan 2018).

But only a few of them are employed as there is a concern over environment pollution/risk/hazard (Santos et al. 2015; Das et al. 2014c; Prasad et al. 2015; Feregrino-Perez et al. 2018).One of them is Silicon nanopartilces, as they possess a positive regulatory effect on plant growth and development when plants are under stress conditions. Likewise, appropriate treatment TiO_2 nanoparticles and carbon nanotubes enhances seed germination in spinach, wheat etc. by increasing water uptake by seeds during drought stress (Khodakovskaya et al. 2009).Quantum dots (QD) are considered to be very tiny semiconductor particles, in the nanoscale range. There is a growing concern about geminiviruses mediated stress (pathogenicity) in plants (Marwal et al. 2013a, b) e.g. sugar beet has been known to challenge with Rhizomania, one of the most critical disease caused by *Beet necrotic yellow vein virus* (BNYVV). The virus is transmitted by the fungal vector *Polymyxabetae* (Keskin) and has been fruitfully detected by QD based sensor (Frasco and Chaniotakis 2009; Algar and Krull 2008; Safarpour et al. 2012). Quantum dot is now being considered for biosensor manufacture for viral disease detection/diagnosis to facilitate the already established management practices (Khurana and Marwal 2016), efficiently help reduce/prevent the biotic stress, through initial diagnosis of volatile compounds secreted upon by plant itself against virus attacks.

6.6 Conclusion

Abiotic and biotic stresses correspond to the negative impact of non-living and living factors on plants. As such their studies need to more and more rely on gene expression. Many crop plants are sensitive to a wide range of (abiotic and biotic), stresses involving various strategic interactions with the host hence directly affecting their growth and yield. This suggests that genes and/or gene classes associated with plant defences against abiotic and biotic stress actin a co-regulatory manner. Despite a large no of abiotic and biotic stress, plants have developed a variety of defences (innate physical and biochemical barriers) (Das et al. 2011; Marwal and Gaur 2017) reflected in their gene transcripts and proteins as shown by numerous studies on the defence and stress mechanisms in plants. Plants have a remarkable ability to cope up with highly variable environmental stresses, but we still need to seek a better strategy for developing stress resistant/tolerant plant species with the

aid of genetic/molecular approach. The work on the abiotic and biotic stress signalling mechanism done previously by the pioneers in the field is particularly significant and beneficial to the agriculture sector which supports the lives of growing population. Thus, understanding the various gene expressions, their molecular mechanisms and finding nanotech solutions, will enable researchers to explore biotic and abiotic stress responses in a better way for the plants which are constantly challenged in the natural conditions.

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Genome Editing: Advances and Prospects

7

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Abstract

There is an urgent need to develop quality crop with improved productivity and wider tolerance to the environmental (biotic and abiotic) stresses for addressing different issues including global water crisis, food security, and climate change effect on agriculture. Traditional lengthy procedures for crop improvement including classical breeding and random mutagenesis will not be able to fulfill growing crop demand in near future. Gene targeting technology is a powerful transformative procedure that permits accurate genetic modification in any genome which relies on a variety of molecular editors. Formation of directed DNA cleavage by ZFNs, TALENs, and CRISPR/Cas9, followed by restoration via the DNA repair system either by NHEJ (non-homologous end joining) or by HDR (homology directed recombination), provides a useful insight of gene function and trait modification. In this chapter, we have described the four available types of genome editing tools; meganucleases, ZFNs, TALENs, and CRISPR systems, and discussed their revolutionary applications in precision molecular breeding and functional genomics research of crops. Furthermore, specific challenges in the plant genome editing and prospects were also reviewed.

Keywords

Genome editing · Meganuclease · ZFNs · TALENs · CRISPR · ABEs

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7.1 Introduction

Plants are the vital source of food for humans and animals, additionally, they are a significant producer of natural chemicals, pharmaceuticals, fuels and other value-added products. Nowadays, plant yield and sustainable production under varying environment are highly needed. Conventional plant breeding was commonly used for enhancing qualities and quantities of traits. Plant breeders explored genetic variation of the primary gene pool of a crop by crossing their elite genotypes to introduce novel alleles. In this procedure, trait linked molecular markers are explored to increase the crop efficiency. Traits are linked with variations and were apeared through spontaneous mutations during evolution and domestication of crop species. Productivity of crops increased around 7% in developed countries where as 30% in developing countries after introducing genetically modified crops (Carpenter 2010). After 1995, only few varieties produced by genetic engineering have commercialized and most of them are herbicide tolerant and insect tolerant. The strict regulation

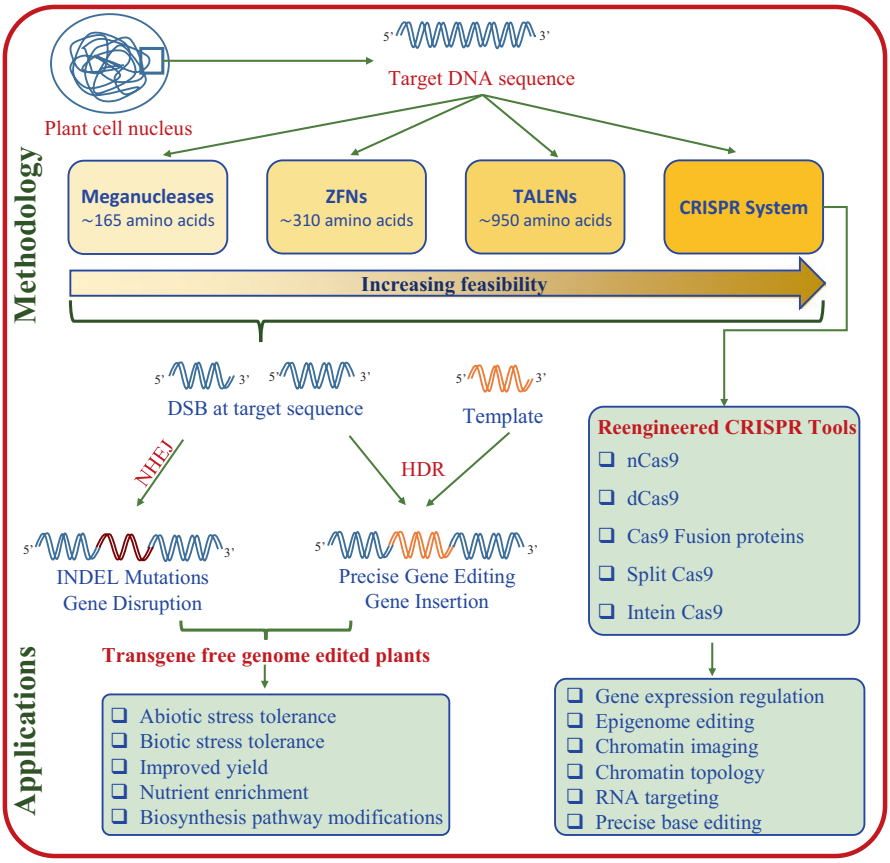

Fig. 7.1 A schematic representation of methodology and application of genome editing

and public skepticism have decreased the interest of researchers to work on GM crops (Armin et al. 2017).

Newly developed genome editing technologies allow a precise gene modifications (Fig. 7.1) by using SSNs (Site Specific Nucleases). Programmable SSNs like meganuclease, ZFNs, TALENs, and CRISPR system facilitate very accurate gene targeting by moving to the cell nucleus and making DNA DSBs (double-strand breaks) at determined target site in genome. Target cell initiates DNA repair mechanism after breakage which may introduce deletions and insertions or large fragment insertions. During non-availability of the homologous template, NHEJ pathway followed for repair of DSBs in which two DNA ends directly ligate together, producing deletions or even hardly insertions (INDEL) at junction of ligated chromosomal ends. INDEL mutations produced during NHEJ repair pathway changes ORF structure or arrangement by frameshift causes changing in protein structure, which produce gene knockout (loss of function). An alternative repair mechanism homology directed repair attained either in the presence of homologous sequences or introducing DNA repair template along with SSNs at target cleavage site. DNA ends produced by SSNs are ligated by homology-directed repair whereas capturing introduced foreign heterologous DNA sequences produced targeted knock in (targeted insertion) plants (Puchta et al. 1993; Bibikova et al. 2001). Nowadays CRISPR, including Cas9 and Cpf1 system is widely used for precise gene targeting in both model and crop plant systems (Gao et al. 2018; Kaur et al. 2018; Li et al. 2018; Zhong et al. 2018). Recently developed ABEs based on dCas9 can precisely modify single base pair in target genome sequence which is a huge breakthrough and opened up a new era in the genome editing (Hua et al. 2018). This chapter describes SSNs viz. meganuclease, ZFNs, TALENs, and CRISPR system and revolutionary applications of CRISPR system in plant genome editing with selected examples in detail.

7.2 Meganucleases

Homing endonuclease enzymes or meganucleases are universal enzymes, and involved in the genomic alteration, reorganization, defense, and repair. Meganucleases identifies14–40 bp long DNA sites which are complex, devoid of any natural site, and absence of that site in host genome for undesirable off-target effect avoidance (Puchta and Fauser 2013; Adli 2018). I-*Sce*I meganuclease recognize 18 bp long sequence and initially purified from yeast mitochondria. The I-*Sce*I ORF incorporates into the mitochondrial 21S rRNA gene by DSB which repaired by copy of the 21S rRNA gene that already contains the I-*Sce*I ORF as an intron, and by this mechanism, it (I-*Sce*I) can spread in the mtDNA (Jacquier and Dujon 1985). I-*Sce*I was used for the first time to confirm applicability of DSB mediated HR in model plant tobacco (Puchta et al. 1993). Meganucleases enzyme system has two major disadvantages. Firstly, lots of meganucleases found naturally has distinctive recognition site. Thus, the possibility of identifying meganuclease that works on preferred target site is less. Second, plenty of created DSBs are restored by

error-prone NHEJ repair mechanism which leads to non-incorporation of exogenously introduced DNA (Adli 2018; Voytas 2013).

Engineering by meganuclease is a challenging job due to its overlapping cleavage and DNA-binding domains. The enzymatic proficiency commonly compromised when amino acid sequences are changed that leads to attaining a new DNA sequence cleavage specificity (Stoddard 2011). Although researchers re-engineered naturally existing meganucleases and significantly improved gene targeting, still very small part of genome can be targeted so far. This enzymes are the tiniest genome engineering reagents typically act as dimers of two indistinguishable subunits (Aminoacid units in single monomers: ~165 in meganuclease, ~310 in ZFN and ~950 in TALEN), that makes easier to deliver in a cell, particularly, if the carrying capacity of a vector is restricted (Smith et al. 2006; Frederic and Philippe 2007; Christian et al. 2018).

7.3 Zinc Finger Nucleases

The zinc finger nucleases (ZFNs) were developed to overcome the limitation of a meganuclease. ZFNs are fusion proteins which include engineered DNA binding domain of eukaryotic transcription factors, zinc finger proteins (ZFPs) and a DNA cleavage unit from *Fok* I restriction enzyme (Urnov et al. 2005; Miller et al. 2007). Precise recognition of long target sequences together with adequate flexibility for retargeting to user-defined sequences is prerequisite of endonuclease to be used as an efficient tool in the genome editing (Klug and Rhodes 1987). ZFNs can be precisely engineered to break fundamentally any dsDNA sequence by altering zinc finger proteins. Zinc fingers proteins (ZFPs) are a basic driver for designing of new DNA binding motifs as this region facilitates the ZFN to bind at distinct target sequences. Binding motif consists of a tandem array of Cys_2-His_2 fingers, which can identify about 3 bp of DNA sequence (Bibikova et al. 2001; Porteus and Baltimore 2003). ZFNs constitute active unit as dimers in which each monomer recognize and bind to half site. Formation of ZFNs dimer facilitated by the *Fok*I nuclease domain that cleaves inside a spacer sequence. Spacer sequence is five to six base pairs in length and separates two inverted half-sites (Cai et al. 2009; Townsend et al. 2009; Urnov et al. 2010).

There are various engineering platform available to design customized ZFNs. Modular assembly was initial free access tool available for designing of a custom ZNF (Wright et al. 2006). This tool is commonly used to develop ZFNs economically by conventional restriction digestion-based sub-cloning systems; though some ZFNs prepared by this method were not very active (Ramirez et al. 2008). Gene correction of *ABI4* and *KU80* genes was successfully performed using a modular assembly approach in a model plant *Arabidopsis thaliana* (Osakabe et al. 2010). Selection based engineering platforms were commonly used to overcome the modular assembly limitations. The oligomerized pool engineering protocol (OPEN) is another openly accessible selection-based tool which design very efficient multi-finger arrays (Zhang et al. 2010). Zinc finger blends recognized by OPEN works

efficiently. ZFN recognition sequence, designed by OPEN, exist probably once at every 200 bp. Endogenous genome editing was successfully carried out in *Arabidopsis thaliana* using ZFNs for *ADH1* and *TT4* genes using the OPEN platform. Context-dependent assembly (CoDA) was recently developed platform by combining features of modular assembly and OPEN. The CoDA has successfully targeted 50% genome sites of *Arabidopsis thaliana* and soybean (*Glycine max*) (Sander et al. 2010; Curtin et al. 2011).

7.4 Transcription Activator-Like Effector Nucleases

Even though ZFNs created considerable excitement as a genome-engineering tool, two research groups identified new technique engineering DNA binding specificities based upon TALEs (transcription activator-like effectors) proteins from *Xanthomonas* phytopathogen (Miller et al. 2010; Boch et al. 2009; Christian et al. 2010). TALEs from *Xanthomonas* precisely recognized one single base (as by ZFNs recognized three bases) in target DNA sequence had encouraged further interest about TALEs. *Xanthomonas* transferred TALEs to plant cells during infection and altered the host plants transcriptome through interaction with sequences present in promoter regions, proficiently mimicking host plants transcription factors (Kay and Bonas 2009; Kay et al. 2007). In TALE protein configuration, a central repeat domain facilitates identification of DNA bases, in which single monomer unit of 33–35 amino acids identifying one base. Two amino acids of the monomers situated at positions 12 and 13 are highly variable also called 'repeat variable di-residue (RVD),' which is accountable for identification of correct target nucleotide. Apart from RVDs all other amino acid composition is highly conserved (Moscou and Bogdanove 2009). Each 34-amino-acid unit folds into a hairpin-like configuration in which 12th amino acid positioned back to stabilize the hairpin, while the 13th amino acid creates a base-specific contact in the major groove of DNA. The last monomer which binds to the 3′-end only contains 20 amino acid residues rather than 34 amino acid (commonly known as half-repeat). Presence of thymidine (T) is necessary in 5′-end of the recognition site which also affects binding efficiency (Miller et al. 2015). The most common RVDs amino acid residues are His-Asp (HD), Asn-Ile (NI), Asn-Gly (NG) and Asn-Asn (NN) which recognize the nucleotides cytosine (C), adenine (A), thymine (T), and guanine (G), respectively (Boch et al. 2009; Deng et al. 2012; Moscou and Bogdanove 2009). Additional guanine-specifying RVDs, namely Asn-His (NH) and Asn-Lys (NK) have been reported to bind guanine exclusively (Cong et al. 2012; Streubel et al. 2012).

Similar to ZFNs, recombinant protein with *Fok*I DNA cleavage motif and TALE modules worked as an effective programmable nuclease and called a TALENs which comprised of NLS (nuclear localization signal). TALENs work in pairs and their recognition sites are selected in such a way that they are situated at opposite to DNA strands and are separated by 12–25 bp of spacer sequences (Abdallah et al. 2015). TALENs can be quickly generated by several methods including Golden Gate assembly method (Engler et al. 2009; Cermak et al. 2011; Zhang et al. 2011;

Sanjana et al. 2012) as well as ligation-based systems (Sander et al. 2011; Reyon et al. 2012). Significant advantages of TALENs over ZFNs is their target site present at every 10 bp on average which is much more repetitive than ZFNs (Curtin et al. 2012). TALENs were successfully used in promoter disruption of *OsSWEET14* gene to increase disease resistance in rice (Li et al. 2012). Disease resistant wheat verity was produced by the knockout of *TaMLO* gene (Wang et al. 2014). Oil composition was enhanced by TALEN mediated knockout of *FAD2-1A*, *FAD2-1B* and *FAD3A* genes in soybean (William et al. 2014; Demorest et al. 2016).

7.5 Clustered Regularly Interspaced Short Palindromic Repeats

Engineering of ZFNs and TALENs system is a complicated process and considered a major constraint for broad acceptance of these enzyme systems by the scientific community. Recently, a class of RNA programmable nucleases has been discovered which is known as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats). This system is considered the most efficient, much easier and more flexible for genome editing. CRISPR has ameliorated the genome editing field because of its robust efficacy and editing efficiency among the existing tools. Currently, CRISPR gene-editing technology is comprised of nuclease enzymes with modifiable targeting activity can be engineered by a short guide RNA (sgRNA). It is worth to note that CRISPR was initially identified as a DNA repeat element in prokaryote later on considered as part of bacterial immune system.

7.5.1 History of CRISPR

In 1987, CRISPR identified incidentally in *E.coli* genetic material during cloning of the *iap* gene, which showed the repeat elements in the genome (Ishino et al. 1987). Later on, these sequences found in some other microorganisms including archaea *H. mediteranii* (Mojica et al. 1995). These repeated elements were reported from prokaryotes only, so far and it was thought that they are not present in eukaryotes and viruses (Ruud et al. 2002). Initially, four CRISPR-associated genes (Cas1-4) were identified from prokaryotes (Ruud et al. 2002), later on, proteins functionally related and similarly structured to Cas were identified and various type of CRISPR system were reported (Haft et al. 2005). Further research into CRISPR proved that some CRISPR spacers are devised from phage and plasmid DNA (Pourcel et al. 2005; Mojica et al. 2005; Bolotin et al. 2005). Initially, their function was unknown, but in 2007, enhanced-acquire resistance was developed in *Streptococcus thermophilus* against a virus through CRISPR by introgressing DNA elements of virus into the CRISPR repetitive sequence of bacteria (Barrangou et al. 2007). Purified Cas proteins digest targeted bacteriophage and plasmid DNA (Garneau et al. 2010) and CRISPR RNAs (crRNAs) act as a guide in the CRISPR system (Brouns et al. 2008).

Collectively, CRISPR/Cas system turned into a directed gene targeting machinery by collectively representing tracrRNA, crRNA, and Cas9 proteins that cut a DNA at specific site (Jinek et al. 2012). In subsequent years, CRISPR/Cas9 system was demonstrated for plant gene targeting by agroinfiltration or protoplast transfection with different repair mechanisms (NHEJ and HDR) in plants such as Arabidopsis, tobacco and maize (Feng et al. 2013; Li et al. 2013; Nekrasov et al. 2013; Xie and Yang 2013; Xiquan et al. 2013).

7.5.2 Basic Mechanism of CRISPR: In Brief

Each intrinsic CRISPR/Cas system consisted of a group of Cas genes which encodes RNA dependent endonucleases, unique noncoding short RNAs called spacers (formed by insertion of invader nucleic acids known as protospacers) and identical palindromic repeats. During cell infection, some spacers were translocated from protospacers to the identical palindromic repeats. These spacers recognize the invaded cells and then trigger the disintegration of invaded cells genetic material. Though the CRISPR/Cas system exists in both bacterial and archaeal genomes, archaeal genetic material contain 87% of CRISPR/Cas as compared to 50% in bacterial genetic material (Makarova et al. 2011; Westra et al. 2014). CRISPR/Cas defense system commonly proceed in three phases. In the first adaptation phase, new spacers are translocated to CRISPR repeats in sequential order. Cas genes and pre-crRNA are expressed in the second (expression) phase and corresponding proteins are synthesized. Cas proteins convert pre-crRNA to mature crRNA. In the third phase, a target region is disintegrated by the combined effort of cr-RNA and Cas proteins (Bortesi and Fischer 2015).The CRISPR motif, commonly known as a protospacer adjacent motif (PAM) is a highly preserved motif of the foreign genome and adjacently positioned in the target sequence for the easy recognition (Barrangou et al. 2007; Brouns et al. 2008; Marraffini and Sontheimer 2008). Cas 9 protein also has specific PAM sequence which contain conserved dinucleotide located at 5′ end of crRNA-binding site for the precise identification of binding site (Jinek et al. 2012). PAM also helps in identification of self DNA from invader DNA (Mojica et al. 2009). In some CRISPR/Cas systems, each Cas orthologues have unique PAM requirement. For example, *S. pyogenes* Cas9 target DNA contained PAM 5′-NGG (Jinek et al. 2012); however, *S. thermophilus* Cas9 required 5′-NNAGAA (Cong et al. 2013; Garneau et al. 2010) or 5′-NGGNG (Gasiunas et al. 2012) as a PAM sequence. The Cas9 of *Neissseria meningiditis* contained 5′-NNNNGATT as a PAM sequence (Zhang et al. 2013).

7.5.3 CRISPR Classification

Till date, various type of CRISPR/Cas system has been identified and each of them have their unique functional and physio-biochemical properties. In recent times, some advantageous single peptide chain CRISPR/Cas system like Cas13a/C2c2 and

Cas12a/Cpf1 have been reported (Abudayyeh et al. 2016; Kim et al. 2016a). CRISPR/Cas systems are distributed in two classes, based on proteins requisite in guide binding domain and target cleavage sites. Class 1 system includes CRISPR RNA binding cascades which are made of multiple subunits (Cas3 or Cas10). In class 2 system, there is a single, multi-domain protein which recognize guide binding site as well as perform target disintegration (Makarova et al. 2015). Because of its unique unaccompanied protein functioning, class 2 CRISPR system has been readily reengineered for gene targeting additionally they generate a DSB which is another plus point (Brouns et al. 2008; Hochstrasser et al. 2014). Class 2 CRISPR/Cas system includes types II, IV, V and VI nucleases (Smargon et al. 2017). Cas9 protein was first identified and characterized widely belongs to type II of class 2 system (Barrangou et al. 2007; Garneau et al. 2010; Jinek et al. 2012). Additionally, it (Cas9) was the first CRISPR system which was applied for gene targeting in bacteria (Gasiunas et al. 2012) and mammalian cells (Cong et al. 2013; Kim et al. 2016a; Mali et al. 2013). Type V DNA-targeting nucleases are known as Cas12a and Cas12b (formerly known as Cpf1 and C2c1, respectively) (Shmakov et al. 2015; Zetsche et al. 2015).Type VI nucleases contain Cas13a and Cas13b (formerly known as C2c2), which are RNA-guided RNA-cleaving nucleases (Abudayyeh et al. 2016; Smargon et al. 2017). As compared to Cas9, Cas12b and Cas12a can process its crRNA without acquiring a trans-activating crRNA (tracrRNA).

Though investigators repurposed various CRISPR/Cas systems for gene editing, type II CRISPR-Cas9 system gained worldwide acceptability for different applications because of its simple PAM sequence (NGG) requirement. However, investigators are still discovering alternative CRISPR systems to recognize more effective nucleases that may have alterations in their PAM requirements, physiology, and substrate requirements. Recently ten new CRISPR/Cas proteins have repurposed for gene targeting. Amongst which Cpf1 from *Acidaminococcus* sp. (AsCpf1) and *L. bacterium* (LbCpf1) are predominantly remarkable (Zetsche et al. 2015; Yamano et al. 2016; Fonfara et al. 2016). Only one single guide RNA required for Cpf1 functioning as compared to Cas9 that require crRNA and tracrRNA. Further, Cpf1 enzyme activity produce sticky ends compared to blunt ends produced by Cas9 (Adli 2018).

7.5.4 Application of CRISPR in a Plant System

CRISPR-Cas systems have been utilized in plants by different mechanisms, such as targeted knock-in, targeted knock-out, single gene knockout, multiplex gene knockout, large fragment deletions and gene knock-out in polyploidy plants for improving yield, disease resistance, herbicide tolerance, nutrient composition, etc. Some tools and web platforms are available for the designing of the CRISPR/Cas system for different organisms, some of them are listed in Table 7.1.

Advancement in CRISPR technology will also boost plant biotechnology and molecular plant breeding program. This new technology allows us to modify or alter multiple gene simultaneously that will enhance crop productivity rapidly and that

Table 7.1 Web tools and online platform available for the designing of CRISPR system

Sl. no.	Software or web tool	Basic platform/ programming language	References
1.	CRISPRdisco https://github.com/crisprlab/CRISPRdisco	Python 2.7	Crawley et al. (2018)
2.	ampliCan https://bioconductor.org/packages/release/bioc/html/amplican.html	R package	Labun et al. (2018)
3.	CHOPCHOP v2 http://chopchop.cbu.uib.no/	Python	Labun et al. (2016) and Montague et al. (2014)
4.	CINDEL http://big.hanyang.ac.kr/cindel/	Python	Kim et al. (2016b)
5.	CRISPR-DO v. 0.1 http://cistrome.org/crispr/	Python	Ma et al. (2016a)
6.	Stupar Lab's CRISPR Design http://stuparcrispr.cfans.umn.edu/CRISPR/	Python	Michno et al. (2015)
7.	CCTop – CRISPR/Cas9 target online predictor https://crispr.cos.uni-heidelberg.de/	Python	Stemmer et al. (2015)
8.	Cas-Designer http://www.rgenome.net/cas-designer/	OpenCL	Bae et al. (2014) and Park et al. (2015)
9.	CRISPR-PLANT https://www.genome.arizona.edu/crispr/index.html	Python	Xie et al. (2014)
10.	COSMID https://crispr.bme.gatech.edu/	HTML	Cradick et al. (2014)
11.	Cas-OFFinder http://www.rgenome.net/cas-offinder/	OpenCL	Bae et al. (2014)
12.	minCED https://github.com/ctSkennerton/minced	Java	Bland et al. (2007)

will increase global food security. Though there are few limitations and prerequisites for gene targeting like knowledge of genome sequences and gene functions. In near future use of CRISPR will increase to explain genome organization, gene annotations and epigenetics like engineering of Cpf1 for gene expression regulation (Agnieszka et al. 2015; Lowder et al. 2015; Tang et al. 2017), gene loci imaging (Chen et al. 2013; Deng et al. 2015; Ma et al. 2015; Ochiai et al. 2015; Tanenbaum

et al. 2014), identifying epigenetic modification (Hilton et al. 2015; Thakore et al. 2015; Vojta et al. 2016), manipulation of chromatin topology (Deng et al. 2014; Hao et al. 2017; Liang et al. 2011; Morgan et al. 2017) and finding association in SNPs recognized GWAS studies and genetic traits (Tak and Farnham 2015). Plants resistance to phytopathogen gemini virus diseases is additional advantage of using CRISPR system if we applied it through viral vector mediated transformation (Ali et al. 2015; Baltes et al. 2015; Ji et al. 2015; Tak and Farnham 2015).

As discussed above, CRISPR technology will also make targeted breeding possible. The CRISPR system has been used to develop a variety of crop qualities including nutrition profile, stress tolerance, productivity and pest and herbicide tolerance (de Toledo Thomazella et al. 2016; Ito et al. 2015; Jinrui et al. 2017; Miao et al. 2013; Sun et al. 2017; Wang et al. 2016a; Xu et al. 2014). After the first report of plant gene editing by CRISPR/Cas9 in 2013, it has been used in a number of species, including arabidopsis, rice, wheat, tobacco, banana, sorghum, tomato, alfalfa, maize, rapeseed, potato, poplar, soybean, barley, switchgrass, moss, wild cabbage, sweet orange, apple, liverwort, grape, robusta coffee, lettuce, cotton, lotus, dandelion, flax, petunia, japanese morning glory, citrus and watermelon (Breitler et al. 2018; Brooks et al. 2014; Chen et al. 2017; Fan et al. 2015; Feng et al. 2014; Gao et al. 2018; Hongge et al. 2017; Iaffaldano et al. 2016; Jia and Wang 2017; Jiang et al. 2013; Kaur et al. 2018; Lawrenson et al. 2015; Li et al. 2015; Lopez-Obando et al. 2016; Mao et al. 2013; Nishitani et al. 2016; Okuzaki et al. 2018; Ren et al. 2016; Sauer et al. 2016; Shan et al. 2013; Shibuya et al. 2018; Sugano et al. 2014; Svitashev et al. 2015; Tian et al. 2017; Waltz 2016; Wang et al. 2015, 2016b; Woo et al. 2015; Yang et al. 2018; Zhang et al. 2016). A comprehensive list of recently developed genome edited plants is given as Table 7.2.

7.5.5 Potential of CRISPR/Cas

CRISPR is emerging as a multipurpose tool which revolutionized not only genome-editing studies but also several other genome and chromatin manipulation efforts, due to its robustness and flexibility. Alternative application areas are mainly possible as a result of the programmable targeting capability of catalytically inactive dead Cas9 (dCas9) (Qi et al. 2013), which cannot cleave target DNA but can still be directed to the target sequence (Jinek et al. 2012). CRISPR-Cas9 contains two catalytic domains (HNH and RuvC) that work together to facilitate DNA DSBs (Yamano et al. 2016). Each catalytic domains cleaves one DNA strand, resulting in DSBs adjacent to PAM sequence at the target site. Markedly, a single point mutation in any of these domains leads to activate a nickase enzyme, while mutations in both domains (D10A and H840A for SpCas9) resulted in total loss of DNA cleavage activity (Jinek et al. 2012). Researchers have now re-engineered these inactive Cas9 variants for a wide range of genome-targeting objectives (Mali et al. 2013; Ran et al. 2013).

Recently, an inventive method called base editing has been developed, based on the CRISPR–Cas9 system for the altering single DNA bases at genomic target sites (Hess et al. 2016; Komor et al. 2016; Ma et al. 2016b; Nishida et al. 2016; Yang

Table 7.2 A summary of the genome edited plants, editing system, and targeted traits

Species	Genome editing tool	Type of edit	Target gene	Target trait	Promoter	References
Actinidia deliciosa	CRISPR-Cas9	Targeted mutagenesis	*AcPDS*	Albino and dwarf phenotypes	*AtU6*	Zupeng et al. (2018)
Arabidopsis thaliana	ABEs	Base editing	*FT*	Late-flowering	*CaMV35S*	Kang et al. (2018)
			PDS3	Albino phenotypes	*YAO*	
Arabidopsis thaliana	CRISPR-Cas9	Targeted mutagenesis	*ISU1*	Fe-S clusters: *de novo* synthesis	*U6*	Durr et al. (2018)
Brassica napus	CRISPR-Cas9	Targeted mutagenesis	*FAD2*	Desaturation of oleic acid	*AtU6*	Okuzaki et al. (2018)
Brassica oleracea	CRISPR-Cas9	Targeted mutagenesis	*BolC.GA4.a*	Biosynthesis of bioactive gibberellins	*AtU6*	Lawrenson et al. (2015)
Citrullus lanatus	CRISPR-Cas9	Targeted mutagenesis	*ClPDS*	Albino phenotype	*U6*	Tian et al. (2017)
Citrus paradisi	CRISPR-Cas9	Targeted mutagenesis	*CsLOB1*	Resistance to citrus canker	*CaMV35s*	Jia and Wang (2017)
Citrus sinensis	CRISPR-Cas9	Targeted mutagenesis	*CsPDS*	Albino phenotype	*CaMV35S*	Jia and Wang (2017)
Coffea canephora	CRISPR-Cas9	Targeted mutagenesis	*CcPDS*	Albino phenotype	*CcU6*	Breitler et al. (2018)
Cucumis sativus	CRISPR-Cas9	Targeted mutagenesis	*eIF4E*	Virus resistance	*CaMV35s*	Jeyabharathy et al. (2016)
Daucus carota	CRISPR-Cas9	Targeted mutagenesis	*F3H*	Anthocyanin biosynthesis	*AtU3*	Klimek-Chodacka et al. (2018)
Fragaria vesca	CRISPR-Cas9	Targeted mutagenesis	*TAA1*	Auxin biosynthesis	*FveU6*	Junhui et al. (2018)
			ARF8			
Glycine max	CRISPR-Cpf1	Targeted mutagenesis	*FAD2*	Fatty acid desaturase	–	Kim et al. (2017a)

(continued)

Table 7.2 (continued)

Species	Genome editing tool	Type of edit	Target gene	Target trait	Promoter	References
Glycine max	TALEN	Targeted mutagenesis	*FAD2-1A* *FAD2-1B*	Monounsaturated oleic acid	–	Demorest et al. (2016)
Glycine max	TALENs	Targeted mutagenesis	*FAD2-1A* *FAD2-1B*	High oleic and low linoleic acid	–	William et al. (2014)
Glycine max	ZFNs	Targeted mutagenesis	*DICER-like*	RNA silencing	–	Curtin et al. (2012)
Gossypium hirsutum	CRISPR-Cas9	Targeted mutagenesis	*GhCLA1*	Albino phenotype	*GhU6*	Pengcheng et al. (2018)
Gossypium hirsutum	CRISPR-Cas9	Targeted mutagenesis	*GhCLA1* *GhVP*	Chloroplast biogenesis	*AtU6*	Chen et al. (2017)
Hordeum vulgare	CRISPR-Cas9	Targeted mutagenesis	*HvPm19*	Grain dormancy	*TaU6*	Lawrenson et al. (2015)
Ipomoea nil	CRISPR-Cas9	Targeted mutagenesis	*EPH1*	Petal senescence	*AtU6*	Shibuya et al. (2018)
Lactuca sativa	CRISPR-Cas9	Targeted mutagenesis	*BIN2*	Increased BR signaling	–	Woo et al. (2015)
Linum usitatissimum	CRISPR-Cas9	Targeted mutagenesis	*EPSPS*	Glyphosate herbicide resistance	*AtU6*	Sauer et al. (2016)
Lotus japonicus	CRISPR-Cas9	Targeted mutagenesis	*LjLbs*	Hairy root and albino phenotype	*LjU6*	Wang et al. (2016b)
Malus prunifolia	CRISPR-Cas9	Targeted mutagenesis	*MpPDS*	Albino phenotype	*AtU6*	Nishitani et al. (2016)
Marchantia polymorpha	CRISPR-Cas9	Targeted mutagenesis	*ARF1*	Auxin biosynthesis	*MpU6*	Sugano et al. (2014)
Medicago sativa	CRISPR-Cas9	Targeted mutagenesis	*SPL9,*	Role in plant development	*AtU6*	Gao et al. (2018)

Musa acuminata	CRISPR-Cas9	Targeted mutagenesis	*MaPDS*	Albino phenotype	*OsU3*	Kaur et al. (2018)
Nicotiana attenuata	CRISPR-Cpf1	Targeted mutagenesis	*AOC*	Jasmonic acid biosynthesis	–	Kim et al. (2017a)
Nicotiana benthamiana	CRISPR-Cas9	Targeted mutagenesis	*NbPDS3* *NbIspH*	Albino phenotype	*AtU6*	Yin et al. (2015)
Nicotiana tabacum	CRISPR-Cas9	Targeted mutagenesis	*bar*	Bialaphos-resistant	*CaMV35S*	Mercx et al. (2016)
			mCherry	Red fluorescent protein		
Oryza sativa	ABEs	Base editing	*OsSPL14*	Ideal architecture and grain yield	*OsU6*	Hua et al. (2018)
Oryza sativa	APOBEC1-XTEN-Cas9	Base editing	*NRT1*	Nitrogen transporter	*OsU6*	Lu and Zhu (2017)
			SLR1	Plant height		
Oryza sativa	APOBEC1-XTEN-Cas9	Base editing	*OsCDC48*	Senescence and death phenotype	*OsU3*	Zong et al. (2017)
			OsNRT1.1B	Nitrate utilization		
			OsSPL14	Grain productivity		
Oryza sativa	CRISPR-Cas9	Targeted mutagenesis	*MOC3*	Rice tiller number regulator	*U3*	Xixun et al. (2018)
			GW2	Grain width controller	*ACT1*	
					UBI1	
Oryza sativa	CRISPR-Cpf1	Targeted mutagenesis	*OsPDS*	Carotene biosynthesis	*Pol II*	Zhong et al. (2018)
			OsROC5			
Oryza sativa	CRISPR-Cpf1	Targeted mutagenesis	*OsPDS*	Albino phenotype	*OsU3*	Li et al. (2018)
			OsSBEIIb	Starch branching enzyme		
Oryza sativa	CRISPR-Cpf1	Targeted mutagenesis	*OsPDS*	Albino phenotype	*ZmUbi*	Tang et al. (2017)
			OsDEP1	Cysteine-rich G protein		
			OsROC5	Leaf shape		

(continued)

Table 7.2 (continued)

Species	Genome editing tool	Type of edit	Target gene	Target trait	Promoter	References
Oryza sativa	CRISPR-Cpf1	Targeted mutagenesis	*OsRLKs* *OsBEL*	DNA binding activity	*OsU6*	Wang et al. (2017)
Oryza sativa	CRISPR-Cpf1	Targeted mutagenesis	*CAO1*	Albino phenotype	*OsU6*	Begemann et al. (2017)
Oryza sativa	CRISPR-Cpf1	Targeted mutagenesis	*OsPDS*	Albino phenotype	*OsU6*	Wang et al. (2017)
Oryza sativa	TALENs	Targeted mutagenesis	*OsBADH2* *OsCKX2* *OsDEP1*	2-acetyl-1-pyrroline biosynthesis	–	Qiwei et al. (2015)
Panicum virgatum	CRISPR-Cas9	Targeted mutagenesis	*tb1a* *tb1b*	Branch architecture Bushy phenotype	*U6*	Yang et al. (2018)
Papaver somniferum	CRISPR-Cas9	Targeted mutagenesis	*4′OMT2*	Biosynthesis of alkaloids (BIAs)	*AtU6*	Alagoz et al. (2016)
Petunia hybrida	CRISPR-Cas9	Targeted mutagenesis	*PhPDS*	Albino phenotype	*AtU6*	Zhang et al. (2016)
Physcomitrella patens	CRISPR-Cas9	Targeted mutagenesis	*PpKAI2L*	Receptors of strigolactones or karrikins	*PpU6* *PpU3*	Lopez-Obando et al. (2016)
Populus tomentosa	CRISPR-Cas9	Targeted mutagenesis	*PtPDS*	Albino phenotype	*AtU3* *AtU6*	Fan et al. (2015)
Populus tremula	CRISPR-Cas9	Targeted mutagenesis	*4CL*	Lignin & flavonoid biosynthesis	*MtU6*	Xiaohong et al. (2015)
Populus trichocarpa	CRISPR-Cas9	Targeted mutagenesis	*4CL1* *4CL2*	Lignin and flavonoid synthesis	*MtU6*	Xiaohong et al. (2015)
Saccharum officinarum	TALENs	Targeted mutagenesis	*COMT*	Cell wall composition	–	Jung and Altpeter (2016)
Solanum lycopersicum	CRISPR-Cas9	Targeted mutagenesis	*SlDMR6-1*	Downy mildew resistance	*AtU6*	de Toledo Thomazella et al. (2016)

Solanum lycopersicum	Target-AID	Base editing	*DELLA*	Plant hormone signaling	*AtU6*	Shimatani et al. (2017)
			ETR1			
Solanum tuberosum	CRISPR-Cas9	Targeted mutagenesis	*GBSS*	Granule-bound starch synthase	*AtU6*	Andersson et al. (2017)
					StU6	
Solanum tuberosum	TALENs	Targeted mutagenesis	*VInv*	Vacuolar invertase	–	Clasen et al. (2016)
Taraxacum kok-saghyz	CRISPR-Cas9	Targeted mutagenesis	*1-FFT*	Inulin biosynthesis	*AtU6*	Iaffaldano et al. (2016)
Triticum aestivum	APOBEC1-XTEN-Cas9	Base editing	*TaLOX2*	Lipid-hydrolyzing enzyme	*TaU6*	Zong et al. (2017)
Triticum aestivum	CRISPR-Cas9	Targeted mutagenesis	*GASR7*	Heat acclimation	*TaU6*	Liang et al. (2018)
			LOX2	Chloroplast lipoxygenase		
			CER9	Elevated amounts of cutin		
Triticum aestivum	CRISPR-Cas9	Targeted mutagenesis	*TaGW2*	Kernel weight	–	Liang et al. (2017)
			TaGASR7	Grain length		
Vitis vinifera	CRISPR-Cas9	Targeted mutagenesis	*IdnDH*	Biosynthesis of tartaric acid	*AtU6*	Ren et al. (2016)
Zea mays	APOBEC1-XTEN-Cas9	Base editing	*ZmCENH3*	Maize kinetochore protein	*ZmU3*	Zong et al. (2017)
Zea mays	CRISPR-Cas9	Targeted mutagenesis	*ARGOS8*	Ethylene sensitivity	*ZmU6*	Jinrui et al. (2017)
Zea mays	CRISPR-Cas9	Targeted mutagenesis	*ALS2*	Chlorsulfuron resistance	–	Svitashev et al. (2015)
			MS45	Male sterility		
Zea mays	TALENs	Targeted mutagenesis	*MTL*	Haploid induction	–	Kelliher et al. (2017)

et al. 2016). The most effective version of base editor comprised of a protein fusion, involving a Cas9 nickase (Cas9–D10A) and a cytidine deaminase that convert C to T (or G to A), together with uracil glycosylase inhibitor (UGI) to prevent any base excision repair of the base change (Komor et al. 2016; Nishida et al. 2016). Existing base editors provide an extraordinary tool for precisely modifying individual nucleotides in a genome. Codon-optimized base editors can carry out site-specific C to T conversions in some plant species (Li et al. 2017; Lu and Zhu 2017; Ren et al. 2017; Shimatani et al. 2017; Zong et al. 2017). Recent breakthrough of base editing showed A to T and G to C base editing in rice, which used recombinant ecTad protein fused to N-terminus of SpCas9 (D10A) nickase to form ABE-P1 (adenine base editor plant version 1) (Hua et al. 2018). It seems that the deamination specificities of base editing in plant cells might be slightly broader than that in animal cells (Zong et al. 2017). Additionally, edited plants do not contain any indel mutations on off-target sites (Zong et al. 2017; Hua et al. 2018), proposing that in plants, this tool is very accurate. Precise base editing provides a powerful tool for creating point mutations in plants to develop traits and characterize gene function. However, not only the desired C but also other Cs contained by the deamination window are transformed by the deaminase (Zong et al. 2017), but future developments may be successful in narrowing down the deamination window to single base pair. Indeed, cytidine deaminase has just been engineered to decrease its window from ~5 to 1–2 nucleotides (Kim et al. 2017b).

7.6 Factors Influencing Genome Editing Efficacy

There are few factors such as genomic sequences, identification of target sequence, structure of donor template, DNA repair pathway management, delivery of gene editing systems, and donor construct design affect the efficacy of genome editing (Songstad et al. 2017; Wang et al. 2017). Designing of a specific target sequence requires in-depth knowledge of genomic sequences and will helps in reducing the off target effects which leads to increase in genome editing efficacy. Currently various method for delivery of gene editing systems used including agrobacterium mediated, viral mediated, particle bombardment, electroporation and PEG mediated (Demorest et al. 2016; Durr et al. 2018; Kim et al. 2017a; Liang et al. 2018). Though suitable delivery method varies from species to species pre-formed RNP complexes preferably delivered by electroporation or particle bombardment and all the delivery methods have their own advantage and disadvantages. Size, complexities and GC content, self-folding properties of donor template affects the efficiency of genome editing. Larger insertion fragments for INDEL mutations, high GC contents and secondary structure forming donor template sequence decrease the efficacy of genome editing. Very important factor affecting the efficacy of genome editing is DNA repair pathway system which play a key role after introducing CRISPR enzyme system in the target plant. Researchers try to take an advantage of homology directed repair which gives efficient and accurate genome editing capabilities. Chances of off-target effects increases by involving NHEJ repair pathway (Songstad et al. 2017; Vojta et al. 2016; Voytas 2013).

7.7 Conclusion and Future Prospects

Multiplex genome editing, which simplifies rapid stacking of multiple traits in an elite variety, will dramatically improve the agronomic traits in crop plants. Genome editing forms an exceptional possibility for plant breeding and is being applied to an ever-increasing number of plant species due to its low cost, precision, and rapidness. It is very apparent that progressively more plants bred with CRISPR technology will be all set for publicizing shortly. However, there are few challenges in plant genome editing, mainly regarding emerging a unified delivery method and making homology-directed repair efficient, which will perhaps involve state-of-the-art engineering of the CRISPR system and manipulation of DNA repair pathways. Moreover, high-throughput CRISPR based whole-genome functional assessment of genes and DNA elements has not demonstrated in plants so far. On the other hand, because of its high proficiency, user-friendliness, and comparatively low cost, CRISPR-based genome editing has been rapidly established as a powerful resource that is revolutionizing plant biology research and crop trait development, similar to that brought about by molecular cloning and PCR technologies. Even though regulatory concerns are not within the scope of this chapter, it is clear that genome-edited plants will feed the fast-growing world population under our ever-evolving climatic conditions.

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Bioinformatics Intervention in Plant Biotechnology: An Overview

8

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Abstract

Bioinformatics relevant to different disciplines of life sciences is associated with management of huge data generated mainly by recent genome sequencing projects. With the advent of technological advancement in sequencing technologies in the recent years several microbial, plants, animals and human genomes have been sequenced. The exponential increase in the sequencing data led to the emergence of appropriate databases meant for storage, retrieval and analysis by scientists from different disciplines throughout the world. In the era of science of "omics" the relevance of the bioinformatics is being realized and several tools, softwares and databases are being developed for supporting biological research in general. Plant Biotechnology has witnessed several innovations over the years and with deciphering of several model and crop plant genome sequences, strategies for crop improvement are being formulated to strengthen the conventional plant breeding. The bioinformatics in plant biotechnology aims to develop crop specific databases, tools for genome analysis, identification of relevant genes and proteins associated with desirable agronomic traits, genome wide identification of transcription factors, functional and structural elucidation of proteins, identification and validation of molecular markers and many more. This chapter summarizes the recent developments in bioinformatics relevant to plant biotechnology in the era of science of omics.

Keywords

Bioinformatics · Genomics · Omics · Plant biotechnology · Tools · Softwares · Databases

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8.1 Introduction

Bioinformatics represents an interdisciplinary computational approach for understanding life processes and focus on formulation of biological databases, analysis of sequences, annotations, modeling and designing of products (Degrave et al. 2002).

The tools of bioinformatics are being applied in diverse fields of sciences mainly emerging from sequencing genomes of animals, plants, microbes and other life forms. The huge data generated is difficult to be maintained manually and needs computational intervention for proper storage, retrieval and analysis. With the increasing sequence information, there has been substantial development of biological databases accessible to users from diverse fields for research purpose. Databases exclusively for genes, proteins, markers, cDNAs, promoters, transcription factors, structures, genome sequences, and many more are emerging with the advancement of bioinformatics in the recent years.

Bioinformatics has drastically influenced the area of plant biotechnology in several ways by using interdisciplinary scientific approach to address the problems visualized in agriculture to attain global food and nutritional security. Efforts have been made to first develop databases of model organisms whose genome sequences were deciphered and get an insight into the molecular biology of life and several common biological processes. With the advancement in technology the science of "omics" relevant to diverse fields emerged and biological databases exclusively for genomics, proteomics, transcriptomics and metabolomics were developed. With the availability of sequence information and their assessment among diverse organisms using several bioinformatics tools, understanding of life at molecular, cellular and developmental level seems to be possible. The concept of system biology is becoming more popular as it involves inputs from diverse fields of sciences to understand the complex life processes. The present trends in research are substantially influenced by the availability of different biotechnological databases designed by the intervention of bioinformatics. Several genome-wide studies are being carried out for characterizing genes, proteins and metabolites and performing comparative assessment among diverse group of organisms. Bioinformatics is playing a significant role in providing appropriate *in-silico* tools for sequence storage, retrieval and comparison and formulation of specific databases for biotechnological research (Gomez-Casati et al. 2017).

Bioinformatics plays an important role in plant science studies in present days. The appropriate use of computers for developing mathematical algorithms to address biological problems based on sequence information exclusively for plants is one of the major developments in plant biotechnology. Several crop specific databases are available for research.

With the aid of bioinformatics tools one can easily characterize several attributes of genes and proteins. The structural and functional annotation of genes based on sequences is routinely performed using tools of bioinformatics. The analysis of regulatory sequences and comprehensive expression profiling using real time data of several genes associated with desirable agronomic traits the genes, revealing the

three dimensional structure of proteins based on protein sequences is also being attempted using relevant bioinformatics tools.

Some of the significant roles of bioinformatics being visualized pertaining to advancement in plant biotechnology is:

8.1.1 Designing Plants with Desired Attributes

Bioinformatics can help to design plants with improved properties such as disease resistance, stress tolerance, plants with enhanced nutritive value once the information related with the corresponding genes is deciphered. The vital role of bioinformatics in biotechnology field is to decipher markers for identification of genotypes and revealing corresponding phenotypes. Several bioinformatics tools and softwares have been designed based on the emerging data from omics based researches and have immense potential to promote research exclusively in model and applied plant species. The availability of several bioinformatics resources and web sites have expedite the research in the area of plant biotechnology by providing a platform for exchange of information among crop scientists (Mochida and Shinozaki 2010). In the recent years several appropriate tools have been developed for the analysis of complex biological sequence data enhancing the accuracy of physical and genetic map preparation prior to genome sequencing. This could be considered to be one of the great outcomes of collaborative research in the area of plant biotechnology and bioinformatics.

8.1.2 For Exploration of Genetic Diversity

Genetic diversity reflects the variation at the molecular level based on variation in the nucleotide or protein sequences among different organisms. By knowing the closely related plants, or wild relatives of today's plants, sexually compatible species having desirable characteristics could be figured out which could be the source for crop improvement. Several appropriate bioinformatics tools have been developed for the accessing the genetic diversity using molecular markers by constructing phylogenetic trees. Efforts are also being made to characterize the wild ancestors of several crops at sequence level to understand the potential of the novel genes coding for desirable traits, which could be adopted for the breeding programmes. With the availability of genome sequences of several crops in the recent years, the bioinformatics tools are extensively applied for comparative genomics studies to reveal genetic diversity.

8.1.3 For the Study of Gene Function

Several projects are now ongoing for genome-wide identification and annotation of miRNA families from sequenced genomes of different crops. With the discovery of

microRNAs in plants, scientists are targeting certain DNA sequences. These small RNA molecules have immense potential to alter plant growth and development. Studies on gene functions are being done by the mutation approach in miRNA molecules by utilizing tools and techniques of bioinformatics.

8.1.4 For Testing, Analyzing and Identifying Plants

With the use of microarray profiles database, scientists are exchanging the information regarding differences in gene expression. Several biological databases based on real time data; exclusively for expression under different stress conditions are available for different crops. This provides an opportunity to get an insight into the novel genes or sequences associated with infestation of stress conditions like drought, disease, or insect and could be then targeted for developing stress tolerant crops.

Therefore, bioinformatics based tools are being developed to expedite the experimental research and not to replace the wet lab experimentation. The information generated by using *in-silico* tools in plant biotechnology are being extensively used to develop appropriate crop specific databases (Fig. 8.1).

The role of bioinformatics relevant to plant biotechnology is being discussed under following subheadings namely;

1. Genome sequencing projects
2. Expressed sequence tags (ESTs) and cDNA clones
3. Small RNAs (sRNAs)
4. Gene identification and sequence analyses

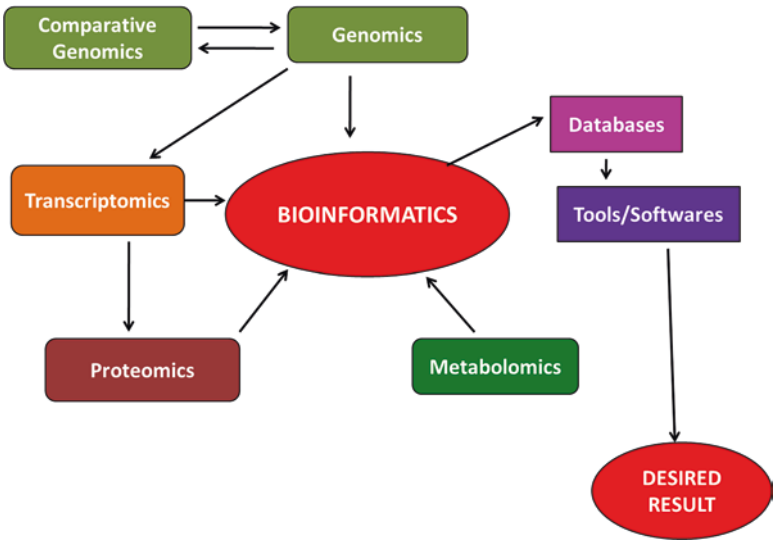

Fig. 8.1 Approaches of bioinformatics in plant biotechnology

5. Phylogenetic analyses
6. Sequence tag-based platforms in transcriptomics
7. Resources in proteomics
8. Resources in metabolomics
9. Transcription factor research

8.1.4.1 Genome Sequencing Projects

Based on innovations in sequencing technologies there has been substantial increase in the genome databases and several plant genomes have been sequenced. The nucleotide or protein sequences resulting from genome sequences needs proper storage, user-friendly methods for the retrieval and development of appropriate tools and softwares for *in-silico* analysis by researchers throughout the world. Further, fast processing systems and softwares are required to handle these enormous data. The large amount of information obtained from sequencing has deepened our understanding and fundamental knowledge of organisms. Innovations in sequencing technologies and bioinformatics intervention are playing a significant role in the advancement of plant biotechnology research. The global transgenic research and development of transgenic crops also popularly known as "Biotech crops" are also maintained by appropriate bioinformatics websites and databases. The information related with biosafety and other issues are also made available by bioinformaticians. A few lists of plant genome databases are shown in Table 8.1.

8.1.4.2 Expressed Sequence Tags (ESTs) and cDNA Clones

An insight into functional attribute of the gene could be easily analyzed by monitoring the mRNA level and comprehensive analysis of mRNA population is a part of transcriptomics research. Expressed Sequence Tags (ESTs) are important for

Table 8.1 List of commonly used plant genome databases

Sl. No.	Plant genome databases	URL
1	Phytozome	https://phytozome.jgi.doe.gov/pz/portal.html
2	Brassica.Info	http://www.brassica.info/info/reference/genome-sizes.php
3	Ensembl Plants	http://ensemblgenomes.org/info
4	GSAD Genome Size In Asteraceae Database	http://www.etnobiofic.cat/gsad_v2/
5	NCBI The National Center for Biotechnology Information	https://www.ncbi.nlm.nih.gov/genome/browse/
6	PGDJ DNA Marker And Linkage Database	http://pgdbj.jp/plantdb/plantdb.html?ln=en
7	Plant DNA C-Values Database	http://data.kew.org/cvalues/CvalServlet
8	Plant RDNA Database	http://www.plantrdnadatabase.com/
9	PlantGDB Genome Browser	http://www.plantgdb.org/prj/GenomeBrowser/
10	PTGBase	http://ocri-genomics.org/PTGBase/

transcriptomics research and are generally derived from gene transcripts converted into cDNAs(Adams et al. 1993). In general cDNA and EST collections lack genomic complexity and are preferred for species with comparatively larger genome sizes. Currently, several plant EST databases are available which have more than 3.8 million sequences representing about 200 species. This database is expanding gradually with the deciphering of several plant genome sequences. These could serve as a powerful tool for analyzing genes and metabolites from different crops. National Center for Biotechnology Information (NCBI)'s dbEST is popular public domain EST database (http://www.ncbi.nlm.nih.gov/dbEST/) representing several crops.

8.1.4.3 Small RNAs (sRNAs)

Small RNA (sRNAs) are known to play significant role in regulating plant growth and development. Substantial efforts are being made to identify and analyse sRNAs from different biological systems using bioinformatics tools. In plants, sRNAs, including microRNAs (miRNAs), short interfering RNAs (siRNAs) and trans-acting siRNAs (ta-siRNAs) plays a significant role in epigenetic processes. It might be also associated with gene networks influencing development and homeostasis (Ruiz-Ferrer and Voinnet 2009). There is a need to identify these RNA molecules and efforts have to be made to decipher the role of of sRNA in gene expression using appropriate genomic technologies (Nobuta et al. 2007; Chellappan and Jin 2009; Thebault et al. 2015). The plant miRNA database (PMRD) (http://bioinformatics.cau.edu.cn/PMRD/) is one of the most popular database comprising several attributes of sRNA including sequence information, secondary structure, target genes and expression profiles. More than 8433 miRNAs from 121 plant species have been included in the PMRD and is substantially increasing with the sequencing of more plant genomes.

A web resource exclusively for siRNA and ta-siRNA regulatory pathways (http://bioinfo.jit.edu.cn/tasiRNADatabase/) known to be associated with plant development, response towards biotic and abiotic stresses and DNA methylation at the TAS locus is yet another contribution of bioinformatics in plant biotechnology. The database ta-siRNAdb includes more than 583 pathways from 18 species and is considered to be one of the largest resource for known ta-siRNA regulatory pathways.

8.1.4.4 Gene Identification and Sequence Analyses

The primary analysis of sequenced genome is to determine the number of probable genes based on several attributes. Using appropriate tools, the genome sequences are firstly subjected to annotation for gene prediction followed by its characterization for several attributes which might reveal the structure and function. Different bioinformatics tools for primary sequence analysis are mentioned in the Table 8.2.

8.1.4.5 Phylogenetic Analyses

The sequence level similarity among different group of organisms can be easily manifested by constructing phylogenetic tree using bioinformatics tools. This phylogenetic tree determine genetic relatedness and reflects the evolutionary relationship (Khan et al. 2014). There exist distance methods, parsimony methods, and

Table 8.2 Bioinformatics tools for gene identification and analysis

Tools	Usage
Genscan, GeneMarkHMM, GRAIL, Genie, Glimmer, ORF Finder	Prediction of introns and exons in a segment of DNA sequence
BLAST	DNA or protein sequence search based on identity
HMMER	Homologous protein sequence search
Clustal Omega	Multiple sequence alignment
Sequerome	Used for sequence profiling.
ProtParam	Physico-chemical properties of proteins
NovoSNP	Single nucleotide variation in the DNA sequence
WebGeSTer	Used to predict the termination sites of the genes during transcription

Table 8.3 Bioinformatics tools for phylogenetic studies

Tool	Feature
MEGA (Molecular Evolutionary Genetics Analysis)	Builds phylogenetic tress to study the evolutionary closeness
PHYLIP	A package for phylogenetic studies
JStree	For viewing and editing phylogenetic trees for presentation improvement
TreeView	Changing the view of phylogenetic tree
Jalview	An alignment editor

likelihood methods for constructing phylogenetic tree. Any of these methods could be applied for making phylogenetic tree and none of these could be considered as perfect. It is always better to construct tree using different methods prior to final assessment. Some of popular bioinformatics tools for phylogenetic analyses are summarized in Table 8.3.

8.1.4.6 Sequence Tag-Based Platforms in Transcriptomics

RNA sequencing (RNA-seq) is an important method for comprehensive gene expression derived from public microarray-based transcriptome data. NCBI's Gene Expression Omnibus (GEO) and the European Bioinformatics Institute (EBI) Array Express are considered to be primary archives of transcriptome data in the public domain. Commonly used databases relevant to transcriptomics are being summarized in Table 8.4.

8.1.4.7 Resources in Proteomics

Proteomics deals with the study of entire sets of proteins revealed in a particular cell or an organism. With the increasing genome sequence information, efforts are being made to study the proteins using several bioinformatics tools providing an insight into the structure and functions. Bioinformatics tools have expedited the proteomics

Table 8.4 Databases for transcriptomics studies

Databases	URL	Analysis
PlantExpress	http://plantomics.mind.meiji.ac.jp/PlantExpress	Public microarray data of rice and Arabidopsis
ATTED II	http://atted.jp/	Arabidopsis ATH1 GeneChip data
Genevestigator	https://www.genevestigator.com/gv/index.jsp	Expression data of various organisms, including Arabidopsis, barley and soybean,
eFP	http://www.bar.utoronto.ca/	Electronic fluorescent pictograp browser provides gene expressionpatterns collected from Arabidopsis, poplar, Medicago, rice and barley
AREX	http://www.arexdb.org/index.jsp	Gene expression patterns of root tissues in Arabidopsis
RICEATLAS, RiceXPro, RicePLEX	http://bioinformatics.med.yale.edu/riceatlas/	Rice transcriptome data of various types of tissues
MOROKOSHI	http://sorghum.riken.jp/	Transcriptome data from *Sorghum bicolour*

research and several new algorithms to handle large and heterogeneous data sets were made for better understanding of different biological processes (Mohammed and Guda 2011). The various tools and servers used in various areas of proteomics are shown in Table 8.5.

8.1.4.8 Resources in Metabolomics

Metabolomics is a rapidly evolving field that generates large and complex datasets. Metabolomics provides comprehensive analysis of metabolites of a particular biological system (Hollywood et al. 2006). The analysis of metabolites of a particular cell of an organism has several advantages in biomarker discovery to reveal disease pathogenesis. Since metabolites plays important roles in biological pathways, appropriate databases and tools could play a significant role in metabolomics research and several new insights of disease and environmental influences could be deciphered. There is a need for bioinformatics tools and softwares for systematic studies in the area of metabolomics.

A typical metabolomics experiment generally has three consecutive phases

(i) Separation of the metabolites using gas chromatography (GC) or high-performance liquid chromatography (HPLC)
(ii) Identification of the metabolites using mass spectrometry (MS) or nuclear magnetic resonance (NMR)
(iii) Analysis of the data obtained

The functional and biological interpretation of metabolomic experiments needs bioinformatics intervention for developing appropriate softwares or tools either for

Table 8.5 Bioinformatics intervention in proteomics studies

Areas of bioinformatics in proteomics	Tools	URL
Peptide mass fingerprinting data	FindMod	web.expasy.org/findmod/
	FindPept	web.expasy.org/findpept/
	Mascot	http://www.matrixscience.com/
	PepMAPPER	bio.tools/PepMapper
	ProteinProspector	prospector.ucsf.edu
Identification with isoelectric point, molecular weight and/or amino acid composition	AACompIdent	expasy.org/aacompident
	AACompSim	expasy.org/aacompsim
	TagIdent	expasy.org/tagident/tagident
	MultiIdent	expasy.org/multiident/multiident
Pattern and profile searches	InterPro Scan	ebi.ac.uk/interpro/search
	ScanProsite	prosite.expasy.org/scanprosite
	HamapScan	hamap.expasy.org/hamap_scan
	MotifScan	myhits.isb-sib.ch/cgi-bin/motif_scan
	Pfam HMM search	pfam.xfam.org
	ProDom	prodom.prabi.fr
	SUPERFAMILY Sequence Search	supfam.org/SUPERFAMILY
	ELM	elm.eu.org
	PRATT	expasy.org/pratt
Post-translational modification prediction	ChloroP	cbs.dtu.dk/services/Chloro
	MITOPROT	ihg.gsf.de/ihg/mitopro
	Predotar	omictools.com/predotar-tool
	PTS1	ppp.gobics.de
	SignalP	cbs.dtu.dk/services/SignalP
Primary structure analysis	ProtParam	expasy.org/protparam
	Compute pI/Mw	expasy.org/compute_pi
	ScanSitepI/Mw	scansite.mit.edu
Secondary and tertiary structure prediction	Homology modeling SWISS-MODEL	swissmodel.expasy.org/
	Modeller	salilab.org/modeler
	Prime	www.schrodinger.com/prime
	CPHmodels	cbs.dtu.dk/services/CPHmodels
	EsyPred3D	omictools.com/esypred3d-tool
	Geno3d	geno3d-prabi.ibcp.fr

(continued)

Table 8.5 (continued)

Areas of bioinformatics in proteomics	Tools	URL
Threading	Phyre (Successor of 3D-PSSM)	sbg.bio.ic.ac.uk/phyre2
	Fugue	mizuguchilab.org/fugue
	Hhpred	hsls.pitt.edu
	LOOPP	cbsu.tc.cornell.edu/software/loopp
	SAM-T08	omictools.com/sam-t08-tool
	PSIpred	bioinf.cs.ucl.ac.uk/psipred
	I-Tasser	zhanglab.ccmb.med.umich.edu/I-TASSER
Quaternary structure	MakeMultimer	watcut.uwaterloo.ca/tools/makemultimer
	EBI PISA	www.ebi.ac.uk/pdbe/pisa
	PQS	pqs.ebi.ac.uk/
	ProtBud	dunbrack.fccc.edu/ProtBuD
Molecular modelling and visualization tools	Swiss-PdbViewer	swissmodel.expasy.org
	SwissDock	swissdock.ch
	SwissParam	swissparam.ch
	pyMOL	pymol.org
	VMD – Visual Molecular Dynamics	ks.uiuc.edu
	CHIMERA	cgl.ucsf.edu/chimera
Protein-protein interaction	SMART	smart.embl-heidelberg.de
	AutoDock	autodock.scripps.edu
	HADDOCK	haddock.science.uu.nl
	MOE	chemcomp.com/MOE-Biologics
	STRING	string-db.org
	IntAct	ebi.ac.uk/intact
	GLIDE	schrodinger.com/glide
	UCSF DOCK6	dock.compbio.ucsf.edu
	ClusPro 2.0	cluspro.bu.edu/publications.php

metabolic pathway mappings and visualizations of a set of metabolites or for the statistical analysis of metabolite annotations.

The bioinformatics tools for the pathway mapping and visualization of metabolites is shown in Table 8.6. Statistical tools for analysis of metabolomics data is summarized in Table 8.7.

Table 8.6 List of bioinformatics tools for pathway mapping and visualization in metabolomics studies

Tools for pathway mapping	
KEGG mapper	http://www.genome.jp/kegg/pathway.html
FMM/from metabolite to metabolite	https://omictools.com/fmm-tool
TabPath/tables for metabolic pathway	https://omictools.com/tabpath-tool
MapMan	http://mapman.gabipd.org/web/guest/mapman
Paintomics	http://www.paintomics.org
Reactome	http://www.reactome.org
MGV	http://www.microarray-analysis.org/mayday
Tools for visualizations of a set of metabolites	
MEGU	http://megu.iab.keio.ac.jp/
KEGGViewer	http://github.com/biojs/biojs
Pathview	https://pathview.uncc.edu/
Cluepedia	http://www.ici.upmc.fr/cluepedia/
WikiPathways App	http://apps.cytoscape.org/apps/wikipathways
IPath	(http://pathways.embl.de)

Table 8.7 Softwares/tools for statistical analysis of metabolomics data

Software/tools for the statistical analysis of metabolomics data		
Ionwinze	LC–MS, GC–MS	http://workflow4metabolomics.org
Galaxy-M	LC–MS	https://github.com/Viant-Metabolomics/Galaxy-M
XCMS Online	LC–MS, GC–MS	https://xcmsonline.scripps.edu
MetaboAnalyst 3.0	LC–MS	http://www.metaboanalyst.ca
MAVEN	LC–MS	http://genomics-pubs.princeton.edu
MZmine 2	LC–MS	http://mzmine.github.io/

8.1.4.9 Transcription Factor Research

In case of plants, transcriptional regulation in response to developmental stages and environmental changes resulting from plethora of biotic or abiotic stresses is being performed by specific transcription factors. The temporal and spatial expression of genes are controlled by the transcription factors (TFs), which recognize specific sequence elements within promoter region resulting either in activating or repressing the activity of RNA polymerase. Transcription factors in general manifest their function by interacting with the basal apparatus either directly or indirectly by a series of co-activators leading to changes in the characteristics of RNA polymerase.

A typical plant transcription factor comprises of four levels of structural organization namely DNA-binding domain, an oligomerization site, a transcription regulation domain and a nuclear localization signal. It is estimated that approximately 5–10% of eukaryotic genes encode TFs and its activity has the potential for alteration in the transcriptome leading to metabolic and phenotypic changes (Riechmann and Ratcliffe 2000; Levine and Tjian 2003; Harbison et al. 2004; Reece-Hoyes et al. 2005; Mitsuda and Ohme-Takagi 2009). It is estimated that more than 100 different DNA binding domains exists and classification of TFs into different families is based on DNA binding domains (Kummerfeld and Teichmann 2006). Bioinformatics intervention in transcription factor research exclusively for plants involves (a) development of appropriate databases for different types of transcription factors both crop specific and transcription factor specific; (b) Genome- wide identification of transcription factor gene families using conserved domains by using appropriate bioinformatics tools; (c) Characterization of identified TFs genes for several attributes like number of introns, physio-chemical features, chromosomal location, motifs analysis, cis-regulatory elements, functional divergence etc.; (d) Insight into the evolutionary aspects of transcription factors based on phylogenetic tree constructions; (e) Structural insight of transcription factors by *in-silico* 3 D structural prediction and validation; (f) Functional elucidation of transcription factor gene families. The role of bioinformatics in plant specific transcription factor research is recently reviewed (Yadav et al. 2016). The overall bioinformatics approach for studying plant specific transcription factor gene families is shown in Fig. 8.2.

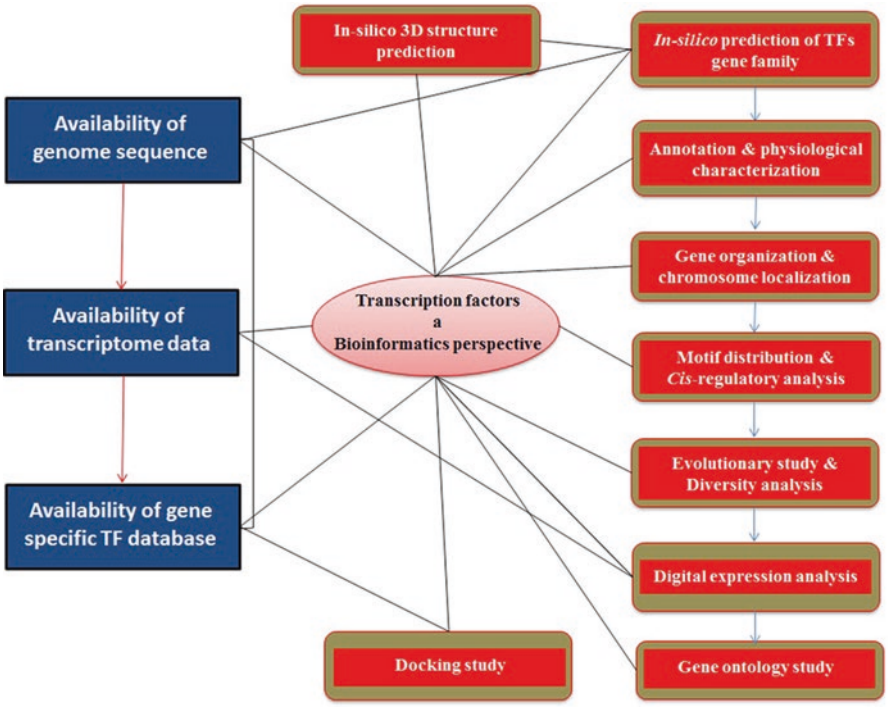

Fig. 8.2 Bioinformatics approaches for genome-wide studies on transcription factors

8.2 Conclusion

The recent advances in plant biotechnology owing to deciphering of genome sequences resulted in exponential growth of sequence data which led to development of appropriate databases and tools for analysis. The bioinformatics research has several dimensions focused on development of (i) new algorithms; (ii) sequence analyses tools; (iii) structural predictions and validations tools; (iv) crop specific and trait specific databases; (v) comparative plant genome assessment tools; (vi) tools for linking genomics, proteomics, transcriptomics and metabolomics datasets; (vii) tools for developing physical and genetic maps; (viii) tools for analysis of NGS data; (ix) tools for analysis of real time data for expression profiling etc. The present computational capabilities require complex operational requirements pertaining to availability, security, data protection, mobility, purpose, sharing and real-time availability which could address the complexity of plant genome sequences.

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Part II

Biofuel & Bioremediation

Plant Oil Biodiesel: Technologies for Commercial Production, Present Status and Future Prospects

9

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Abstract

Emerging environmental impacts and related health issues, the continuous hike in price of vehicle fuels in sync with the depletion of fossil fuels have urged considerable research to move on alternative fuel sources and there comes the word "Biofuel". Biofuel is gaining importance and has been a major subject of research for scientists worldwide. Attention has been given to biofuels in various countries because of its positive impacts on environment and in response healthy manpower. One of the most important and concerned topic of biofuel is biodiesel.

Keywords

Biodiesel · Plant oils · Bio diesel properties · Catalysts · Transesterification · Advantages & disadvantages of biodiese

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9.1 Introduction

Emerging environmental impacts and related health issues, the continuous hike in price of vehicle fuels in sync with the depletion of fossil fuels have urged considerable research to move on alternative fuel sources and there comes the word "Biofuel". Biofuel is gaining importance and has been a major subject of research for scientists worldwide. Attention has been given to biofuels in various countries because of its positive impacts on environment and in response healthy manpower. One of the most important and concerned topic of biofuel is biodiesel.

Biodiesel is a renewable replacement for fossil diesel with a high potential of clean burning fuel improving the environment and can be used as a substitute for fossil diesel without any refitting in existing vehicle engines. Biodegradable totally combustible and non-toxic biodiesel is having low risk rate on spillages as compared to fossil diesel spillages. Biodiesel has many chemical characteristics which make it as a safe fuel to be used. A higher flash point of biodiesel makes it less harmful and toxic in nature. Furthermore, we will discuss more properties of biodiesel as we move towards the analysis report of biodiesel in the later part of the chapter. Biodiesel is also having negative impacts but apart from negative impacts biodiesel is having many benefits too and one of the most important is that this fuel produces no carbon in the form of carbon dioxide or carbon monoxide and hence, can be described as a **'carbon balancer'** i.e. it is completely combustible.

We can produce biodiesel from plant oil, microalgal oil, animal oil/fats, tallow and waste cooking oil. However, presently 95% of the biodiesel is produced from edible oil and which in turn having many negative impacts which will be discussed later in the chapter. The production process used to convert these oils to Biodiesel is termed as trans-esterification. Comparative parallel studies on different trans-esterification methods are presented in this chapter.

Ester exchange or trans-esterification is the basic chemical reaction in the conversion of plants oil to Biodiesel. This chemical reaction converts esters from long fatty acid chains into mono alkyl esters and that is why chemically, biodiesel is a fatty acid methyl ester. Plant oils are trans-esterified by heating them in consortium of large amount of anhydrous methanol and an acidic or basic reagent as catalyst. During the production process a catalyst usually used to increase the rate of reaction and yield. In a trans-esterification reaction, the reaction equilibrium is shifted to the right side to produce more ethyl esters by using larger amount of methanol to get the high yield of product (Leung and Guo 2006). Various plants oil trans-esterification methods are currently used in the production process of biodiesel fuel and as an alternative fuel, plant oil is one of the promising renewable fuels.

However, the high cost of production is the major hindrance in biodiesel production and its commercialization at large scale; the biodiesel production from plan toil or animal fat is far more overpriced than petroleum-based diesel fuel (Demirbas 2008). Since the cost of feedstock amounts approximately 70–95% of total operating costs at a biodiesel plant so this has become a great concern to biodiesel producers and investors of market in biodiesel. Compared to edible vegetable oils, the cost of waste plant oils is anywhere from 60% less to free, depending on the source and availability of the raw material.

Biodiesel is preferable and superior to fossil diesel in terms of sulphur content present, aromatic content and flash point. Biodiesel is completely combustible sulphur free and non-aromatic fuel while fossil diesel can contain up to 500 ppm $SO_2$and 20–40 wt. % of all aromatic compounds present. The finding leads to an advantage and could be a best solution to cut down the pollution and there effects since transport increases exponentially and became the most important contributor of the total gas emissions. Amongst vehicle fuels, diesel is responsible for black smoke particulate together with SO_2 emissions and contributes to a one third of the total transport generated greenhouse gas emissions. Properties of being non-toxic, renewable, completely combustible and biodegradable draw attention to use biodiesel as alternative source of fuel with significantly lower exhaust emissions of particulate matter and green-house gases. Therefore, biodiesel being environmentally friendly with high energy product shows great potential to be used as substitute fossil diesel.

9.2 Plant Oil: Physical and Chemical Properties

Plant oils are triglycerides extracted from plants. Being non polar in nature, these oils are neutral liquids exhibiting the property of high-viscosity at ambient temperatures. In general, oils are hydrophobic and lipophilic in nature. In present, biofuel is the most available alternative form of fossil fuel and hence, the biodiesel is the renewable form of energy for the fossil diesel. So for higher production one should understand and have knowledge about the raw material and which is plant oil in this case. Before production, manufacturers should be aware about the physical and chemical properties of plant oil and its availability and ways to enhance the production of raw material. Main properties to be studied are physical and chemical because raw material has to undergo various chemical and mechanical processes in the production plants. Before using it in diesel engines, it is well required to set the specifications for biodiesel to be used in diesel engines. It is required to consider the high variability of the chemical and physical characteristics of different plant oils and in few cases of oils derived from the same plant species. Generally, these products contain 98% of triglycerides while the remaining 2% consists of phospholipids and other types of hydrocarbons. Glycerides are the products from a molecule of esterified glycerol with molecules of fatty acids, characterised by the variable number of carbon atoms and by the different chemical configuration. The different chemical structure leads to the variability of the characteristics of the different plant oils, and consequently the physical properties of the oil itself (Toscano and Maldini 2007).

According to the literature (Knothe and Steidley 2005), the viscosity of oils is directly related to the level of unsaturation and the length of the fatty acid chains. The viscosity decreases when there are an increased number of double bonds and advances with an increase in the length of the hydrocarbon chain and according to the level of polymerisation in the oil.

Auld, was one of the first researchers to give an equation expressing the relationship between the effect of the quantity of double bonds (measured by Ni) and the length of the hydrocarbon chain on viscosity (measured by Ns) (Bettis, Peterson

et al. 1982). Valdes and Garcia in 2006 verified that using the Auld's equation viscosity is indirectly proportional to the number of double bonds present and to the length of the hydrocarbon chain. The rise in the viscosity of plant oil subjected to quick heating is determined by breaking the double bonds and a gradual reduction in the level of unsaturation (Valdés and Garcia 2006). Configuration of the double bonds is directly related to the viscosity (the cis configuration shows lower viscosity than the trans configuration) while the position of the double bonds and the length of the hydrocarbon chain has less effect.

Many other works in literature (Abramovic and Klofutar 1998) also proved relationships between viscosity and chemical parameters such as Ni and Ns, or more precisely the acidic composition of triglycerides. The viscosity of the plant oil is very closely dependent on the temperature. A non-linear reduction in viscosity was found with an increase in temperature and so large number of equations has been given by many scientists but the equation that best explains the relation between temperature and viscosity is found in Eqs. (9.1) and (9.2):

$$\ln v = a + b / T + c / T^2 \tag{9.1}$$

$$\ln v = a + b / T + c.T \tag{9.2}$$

where,

ν = kinematic viscosity at temperature T
a, b and c are constants

Dermibas in 1998 have given a relation between calorific value and the chemical structure of oil which is expressed by a mathematical equation which also defines the correlation between Ns and Ni and HCV (Demirbaş 1998).

The equation is:

$$\mathrm{HCV} = 49.43 - \left[0.041(\mathrm{Ns}) + 0.015(\mathrm{Ni})\right] \tag{9.3}$$

On increasing the values of Ni and Ns, the HCV gradually decreases proving that energy content increases with increasing length of the chain (increasing number of carbon atoms), but declines when cumulative amount of carbon decreases with respect to the oxygen (Toscano and Maldini 2007).

9.2.1 Edible Plant Oil

The edible plant oils include *Theobroma cacao* (palm oil), *Cocosnucifera* (coconut oil), *Helianthus annus L.* (sunflower oil), *Sesamumindicum* (gingelly oil), Rapeseed (Brassica napus), Canola oil, groundnut oil, mustered oil, ricebran oil, safola oil, cottonseed oil, olive oil and soybean oil (Glycine max). The percentage of oil yield from the plants is itself a key factor in the production of biodiesel and is inversely

proportional to the cost of production. According to the studies, palm oil is having highest oil yield as compared to other edible plant oils which is approximately 5000 Kg/hec (Toscano and Maldini 2007). The composition of the oil itself defines its suitability to be used for biodiesel production and will also define the properties of biodiesel produced. The composition for all the edible oils are almost same except few fatty acids.

Soybean oil has a high content of protein around 35–40% that contains all the essential amino acids needed for human growth and development. Apart from that, soybean oil and rapeseed oil also has high content of linoleic acid, with 51% and 22.3%, respectively and also contains proteins and polyunsaturated fatty acids that are essential fatty acids required by humans which are helpful in lowering down the risk of heart diseases. The oils of these plants are even rich in number of polynutrients such as beta-carotene, alpha carotene,, vitamin-E, lycopene, tocotrienols and other carotenoids like that of palm oil. These poly-nutrients act as an anti-cancer agents because of anti-oxidants properties they are having.

9.2.2 Non-edible Plant Oils

Similar to edible oils, the oil yield from the plants is important to the production cost. Higher the oil yield lower will be the production cost. Jatropha (*Jatropha* curcas), rubber seed (*Ficus elastica*), castor (Ricinus communis), Indian beech (Pongamia pinnata) and sea mango (Cerberamanghas) are some of the plant species used for biodiesel production. Among them, Jatropha produces the higher percentage of oil yield followed by *P.pinnata* and castor plants. However, the oil yield in *P.pinnata* is not stable and highly depends on plantation and extraction factors involved in production processes.

The composition of fatty acids for edible as well as non-edible plant oils is almost comparable except castor oil. In all the non-edible oils castor oil has the most unique composition with approximately 89.5% ricinoleic acid. And, is the reason that ricinoleic acid is also known as castor oil acid, an unsaturated fatty acid which is soluble in most of the organic solvents.

In comparison to edible oil, non-edible oils like jatropha, castor, Indian beech, rubber seed and sea mango contains toxic compounds in the oil and due to this they are not suitable for human consumption. Curcin is the main toxin of jatropha while purgative is another toxin found mainly in seeds, sap and fruits of jatropha and purgative which are found mainly in the seeds, fruits and sap (Toscano and Maldini 2007). The toxic compound of the seed is transferred in to the oil after extraction process. Ricin is a phytotoxin present in castor plants which is a water soluble protein concentrated in the seeds, while the toxin in rubber seed oil is cyanogenicglucoside that yields poisonous prussic acid (HCN) because of an enzymatic reaction. Sea mango tree is a well-known ‘suicide tree’ due of its highly poisonous nature. The leaves and fruits of sea mango contain the powerful cardiac substance called

cerberin (glycoside), which is extremely poisonous if ingested (Toscano and Maldini 2007). The seed of P. pinnata contains pongam oil which is bitter and non-edible due to the presence of flavonoid constituent, pongamiin and karajiin and is usually used as a fish poison.

9.3 Process of Biodiesel Production from Plant Oils

The production of biodiesel is the series of chemical reactions involved in trans-esterification. In general, trans-esterification is used to define the chemical reactions through which an ester is transformed into another ester by interchanging the alkoxy-group. In the field of biodiesel production many researchers in their recent publications also defined the trans-esterification as the synonym used for alcoholises of carboxylic esters (Schuchardt, Sercheli et al. 1998). It is an equilibrium state and the trans-formation occurs by mixing of the reactants. Addition of a catalyst (use of strong acid) acts as a positive inducer which accelerates the rate of reaction. Accordingly, the yield of an ester increases with an increase.

A successful trans-esterification reaction is signified by the separation of the methyl ester (biodiesel) and glycerol layers after the reaction time. The products of the trans-esterification reaction are the biodiesel and glycerol. After the trans-esterification reaction and the crude heavy glycerine phase is separated and the product is left with a crude light biodiesel phase. This crude biodiesel requires some purification prior to use in engines. The basic process of plant oil extraction till biodiesel purification is similar to all edible as well as non-edible plant oils but the difference lies in the type of the process and the catalyst used. There are different methods known till now for biodiesel production and these are:

1. **Direct use and blending**: It is defined as the direct use as a diesel fuel or by blending it with diesel fuel. Easy portability because of its liquid nature is its advantage. The biodiesel produced by this method is having high viscosity, lower volatility and reactivity of unsaturated hydrocarbon chains which makes it unsuitable to use in engines (Kaya et al. 2009).
2. **Micro-emulsion**: It is defined as a colloidal equilibrium dispersion of optically isotropic fluid microstructures with dimensions generally in the 1–150 nm range formed spontaneously from two immiscible liquids and one or more ionic or non-ionic amphiphiles. The biodiesel produced by this way is having better spray pattern during combustion with lower fuel value. But it is also having a disadvantage of lower energy content by having a lower Cetane number (Sahoo and Das 2009).
3. **Thermal Cracking (pyrolysis):** It is the conversion of long chains and saturated substance for the production of biodiesel. The biodiesel produced is chemically similar to the fossil fuel. Due to energy intensive it is having high cost (Alonso et al. 2008).

4. **Trans-esterification:** it is a reaction between a fat or oil with an alcohol in the presence of a catalyst to produce ester and glycerol. The biodiesel produced by trans-esterification is having high cetane number with lower emissions and higher combustion efficiency. It is also having high renewability rate with a single disadvantage of disposal of waste product and having no negative effect on engines while using the biodiesel produced as a fuel (Zubr 1997).

The use of suitable catalyst in the reaction plays an important role in the yield of biodiesel production. Alkalis, acids and enzymes are generally used as catalysts for biodiesel production. Enzyme catalysts have come into use recently because of its some positive role. The use of an enzyme as a catalyst for biodiesel production prevents soap formation and hence simplifies the process of purification. Enzyme-catalyzed reaction is good over base and acid catalyzed reaction but it is costly process. After the production of biodiesel, it is contaminated with residual catalyst, water, unreacted alcohol, free glycerol and soaps, the crude biodiesel produced needs to be purified using different techniques (Toscano and Maldini 2007). The lists of different techniques used for purification of crude biodiesel are given:

Water Washing This technique is very effective as both glycerol and alcohol are soluble in water. It can also remove the left residues of sodium salts and soaps. Washing is done with warm distilled water or softened water. The use of warm water prevents the precipitation of saturated fatty acids present and retards the formation of emulsions. Softened water removes the traces of calcium and magnesium salts contamination and also neutralizes the remaining alkali catalysts. After few washings, the water phase becomes clear indicating that the contaminants have been completely removed. Now the biodiesel and water phases are separated using separation funnel or centrifuge (De Oliveira et al. 2004). Molecular sieves and silica gels are also used to remove the water from the biodiesel because of the immiscibility of water and biodiesel. The remaining wastewater can be removed from the biodiesel by passing the product over heated Na_2SO_4 overnight and then removed by filtration.

Dry Washing Researchers also used dry washing by using an ion exchange resin or a magnesium silicate powder replacing water to remove the contaminants. The use of these two materials lower down the level of free glycerol and is also effective for removing soaps. Both the ion exchange process and the magnesol process are advantageous of being waterless and thus eliminate many of the problems that persist in water washing. Themagnesol process has a better effect on the removal of methanol than the ion resins but none of the products from this process fulfil the limits of EN Standard.

Membrane Extraction With advancement in the purification methods the researchers proved that the use of hollow fibre membrane extraction like polysulfone can remove the contaminants giving better efficiency. In this technique, a hollow fiber membrane of 1 m long and 1 mm diameter filled with distilled water which

is immersed into the reactor. The crude biodiesel produced is pumped into the hollow fiber membrane with a flow rate of 0.5 ml/min and 0.1 MPa as operating pressure. After this step, biodiesel is passed over heated Na_2SO_4 and then filtered out to remove any remaining water (Abramovic and Klofutar 1998). This technique avoids the formation of emulsions during the washing step which in turn decreases the loss during the refining process. The biodiesel obtained is 90% pure and all other properties are specified by ASTM standards and it is one of the very promising methods for purifying biodiesel. For commercial fuel, the finished biodiesel must be analysed using sophisticated analytical equipment to ensure that it meets international standards.

Apart from the above discussed conventional processes for biodiesel production other processes are also there and these are:

1. **In situ biodiesel production**: In this method the oilseeds are directly treated with methanol at optimum temperature and pressure in which the catalyst has been mixed to achieve the trans-esterification which means that in this method oil is not extracted from the oil seeds prior to the esterification reaction. In this type of production the oil seeds are dried before to be used in situ trans-esterification to reduce the consumption of alcohol. At the end of the reaction two layers are formed. The upper layer consists of crude biodiesel and lower layer of alcohol that can be recovered easily. The crude biodiesel in the upper layer is washed several times to separate out the contaminants, after washing it is dried over anhydrous sodium sulphate and finally filtered to get the pure biodiesel. The final diesel obtained conform the ASTM standards. This process was first given by Harrington and D'Arcy Evans in 1985 (Harrington and D'Arcy-Evans 1985).
2. **Biox co-solvent process**: This process was developed by Boocock and his group in 1996. In this process, esters are formed by triglycerides through selecting the co-solvents that are capable of generating one phase oil rich system. Co-solvents are used to overcome the slow reaction time because of low solubility of alcohol in the triglyceride phase. Hence, the co-solvent used should be able to make the alcohol soluble like tetrahydrofuran (THF). At the end of the reaction the phase separation of glycerol and biodiesel is clean and excess of alcohol and THF can be removed in a single step (Demirbas 2008). Co-solvents used should be removed completely because they are hazardous and toxic in nature. The advantage of using this process is that it uses inert, recoverable so-solvents in single step at optimum temperature and pressure and so no waste residues are present in biodiesel and glycerol phase. Unlike, in situ process it can be used for waste cooking oils and animal fats. However, recovery of excess alcohol in this process is difficult due to the melting point which is almost equal methanol and THF co-solvent.
3. **Supercritical alcohol process**: It is known that when a gas or fluid is subjected to temperatures and pressures in excess to its critical point, a number of unusual properties take place and these properties are used in this process. In such condi-

tions, a liquid and gas phase no longer exists and only fluid phase is left. Therefore, a non-catalytic supercritical method has been developed. The lower value of dielectric constant of methanol in supercritical state proves to solve the problems associated with the two-phase in a mixture of methanol and triglycerides by forming single phase which in turn reduces the reaction time. This process is carried out at high pressure and temperature. After the reaction the gas is released out and the product is collected. The other contaminants are removed by using methanol. This process is advantageous over alkali catalysed process in reaction time and final purification (Demirbas 2008).

9.3.1 Homogenously Catalysed Trans-Esterification Process

Homogeneously catalyzed trans-esterification of plant oils via one- and two-step processes is the most recent research subject on the FAME synthesis and is the most frequently accepted industrially biodiesel production. The raw material characteristics such as its FFA content and fatty acid composition influence the selection of the biodiesel production process.

9.3.1.1 One-Step Process

The FFA content or acid value in the feedstock defines the choice of use between base and acid. The presence of acid content in the raw material is influenced by many factors like the source of the oil, mechanism behind its storage and the way of cultivation. In comparison to edible oils the non-edible oils contain high amount of FFA that limits the use of base catalysts in the production process and according to literature there is a wide range of acid value for non-edible plant oils. Soap formation is there by a reaction of FFAs from raw material in the presence of base catalyst that reduces the FAME yield, causes the catalyst loss and also complicates the process of separation of phases. Hence, base catalysts are mostly used with the plant oils having low value of FFA content. If the FFA content of oil is high then esterification is done using acid catalyst to lower down the free FFA content followed by transesterification using base catalyst (Banković-Ilić et al. 2012).

The type of the catalyst with optimal reaction conditions and examples are discussed above for homogenously catalysed base and acid esterification reactions. The reaction occurs in the presence of methanol or in some cases it is ethanol at temperatures below the boiling point of ethanol. Literature proved that 90% of ester yield is achieved in this step independently of the catalyst, alcohol and the type of the plant oil. Sulphuric acid and alkali hydroxides are the most used catalysts in the process of trans-esterification (Banković-Ilić et al. 2012). Using homogenous catalysts has many disadvantages like operating problems are due to the hazardous nature of alkali hydroxides. After the production process, the product quality is achieved by the various steps of purification steps removing the large amount of waste by products which are not favourable for the environment and requires a proper treatment before disposal into the environment. As a result the overall production cost increases and that is why new methods have been developed and it is

considered as one of the traditional method in biodiesel production (Banković-Ilić et al. 2012).

The base catalysed reactions are highly catalytically active and that is why in low cost high quality biodiesel is produced in less time. But use of base catalyst is also having a drawback that they are not capable of converting FFA to alkyl esters and reduces the biodiesel yield, prevents glycerol separation due to the soap formation. The high yield of biodiesel is achieved in many cases irrespective of oil source and alcohol like in the castor oil trans-esterification using sodium ethoxide as a catalyst high molar ratio (16:1) of alcohol and oil, the ester yield achieved was 99%.

Similarly, in the case of jatropha and tobacco plant oils the ester yield of 98% was achieved. On the contrary, very low biodiesel yield were also reported for jatropha and in sea mango oils that are having a high FFA content. Most of the researchers used methanol for biodiesel production as compared to ethanol because methanolysis is faster than ethanolysis and FAME yields are higher than FAEE yields (Meneghetti et al. 2006). However, for some oils like that of jatropha the mixture of methanol and ethanol is used to increase the yield of biodiesel. And in this way the property of oil that it is more soluble in ethanol than methanol is used up. Also, mixed esters are having better solubility additives than methyl esters. The initial catalyst concentration is a very important factor determining the TAG conversion degree. The optimal amount of the base catalyst is about 1% based on oil weight, although some researchers have reported both slightly lower catalyst concentrations such as 0.5%, 0.7% or 0.8% NaOH, and higher catalyst concentrations such as 3.3% NaOH or 6% NaOH.

The acid catalyst are known to have some important advantages over base catalysts such as tolerance level and less sensitivity for the high value of FFAs presence (Koh and Ghazi 2011) and the possibility of completion of simultaneous reactions of esterification and trans-esterification. The sulphuric acid, hydrochloric acid and phosphoric acid are the most used acid catalysts.

The yield of biodiesel produced is around 90% in many studies related to the use of acid catalyst for the biodiesel production from plant oils but longer reaction time are needed as compared to the base catalysed reactions at optimum temperature. The biodiesel production is dependent on several factors like type of the oil content, catalyst quantity and type, and reaction time.

9.3.1.2 Two-Step Process

Both base and acid catalysts are used in two-step (acid/base) processes for biodiesel production from plant oils with a high FFA content to take the advantages of both catalysts. The two step process consists of pre-treatment process in which acid catalyzed FFA esterification for reducing the FFAs below 1% and then the base catalyzed TAG alcoholysis process which is an effective way to achieve higher biodiesel yield within a short reaction time at optimum reaction conditions, compared to one-step process. The problem of slow reaction is overcome and the soap formation is eliminated by using acid catalyst in the first step of the process. The only disadvantage of the two-step trans-esterification process in the biodiesel production from plant oils is the higher production cost as compared to conventional, one-step

process. Important factors affecting the acid value in the first and the ester yield in the second step are the type of oil content and alcohol, alcohol:oil molar ratio, catalyst concentration, reaction temperature and reaction time (Banković-Ilić et al. 2012).

It is also shown that with the progress of the reaction up to the optimal reaction time, the FAME yield increases rapidly. In the acid catalyzed pre-treatment, when the reaction time is longer than the optimal one, the physical appearance and colour of the oils become darker. Also in the base catalyzed process at temperatures above 60 °C causes excessive methanol loss due to evaporation and reduces the overall ester yield and hence, increases the production cost (Sharma and Singh 2010).

9.3.2 Heterogeneously Catalysed Trans-Esterification Process

Uses of heterogeneous catalysts over other catalysts are advantageous and promising for biodiesel production as they could be operated in continuous processes and yields high quality products. These catalysts are reusable and are more effective than acid catalysts and enzymes. Most importantly they are environmental friendly and while using these catalysts saponification will not produce soaps. However, apart from advantages there is a major disadvantage of using heterogeneous catalysts and that is a low reaction rate caused by diffusion limitations in the three-phase (oil–alcohol–catalyst) reaction mixture and so they can be modified to increase the activity and self-life of catalysts. Large numbers of heterogeneous catalysts are reported in the literature (Sanjay 2013). The use of heterogeneous catalysts can be through one-step process or two-step processes depending on the type of raw material.

9.3.2.1 One Step Processes

One-step process is a base-catalyzed trans-esterification process which is used where oily feedstock contains less than 1% FFAs with no or very less impurities. Simplified method of biodiesel separation and purification using heterogeneous (solid) catalysts makes them more environmental friendly and also reduces the waste water content during the production. Recent researches have been focusing on low cost and eco-friendly heterogeneous catalysts having high catalytic activity. The catalytic activity depends on specific surface area, its nature, pore size and volume of active site. The steps of washing, drying, crushing and calcinating at very high temperatures are required in the preparation of these catalysts. In one-step process being continuous process development the catalyst reusability is higher (Veljković et al. 2015). For example Cao and hydrotalcite with Mg/Al molar ratio 3:1 can be used for 10 runs even after washing and calcination and 8 runs after washing with ethanol without any loss in FAME yield. The best example of reusability is seen in the SiO2.HF catalyst which is reused 30 times without regeneration and FAME yield loss.

9.3.2.2 Two-Step Processes

The two-step process is also known as acid-catalyzed trans-esterification reaction which is required for the oily feedstock containing high FFA content, water and impurities. The acid catalyzed trans-esterification process is preferable for biodiesel production from the feedstock rich in FFA content. However, the completion of this process requires relatively high temperatures with long reaction time and equipment made from non-corrosive materials. To overcome these problems, the two-step process is used in which pre-esterification using acid catalyst is followed by base catalyzed trans-esterification for the conversion of FFAs and TGAs into esters respectively. Due to the reversibility of both the reactions, an excess of alcohol is required to force the forward reaction. So a need for a bi-functional catalyst that can catalyze the both esterification and trans-esterification reactions at the same time and hence using a bi-functional catalyst it can be done in a single step (Veljković et al. 2015).

According to the best informational studies available, a complete heterogeneous two-step process has not been developed yet and a step is still homogeneous in nature. Still the research is going on for the preparation of low cost catalyst to reduce the total production cost (Veljković et al. 2015).

9.3.3 Enzyme Catalyst Trans-Esterification Processes

Some of the recent research use enzymes in place of catalysts for biodiesel production. Lipases are well-known enzymes that are used to catalyze both esterification and trans-esterification reactions that have a high catalytic activity in water free media. Due to this property the reaction is carried out in non-aqueous conditions like solvent-free systems, gaseous media, supercritical fluids and organic and ionic liquids by making an enzyme immobilized using carriers. Apart from the drawback of high cost of enzymes it is having most advantageous process of simultaneous catalysis of both the reactions TAG alcoholysis and FFA esterification with easy recovery of glycerol and minimal amount of water produced. The origin of lipase is the key factor determining ester yield for example *Chromobacteriumviscosum*, *Candida antractica*, *Mucormiehei*, *Pseudomonas cepacia* and *Rhizomucororyzae* are mostly used in non-edible plant oil trans-esterification reactions. For biodiesel production high concentration of enzymes are required which are mostly immobilized using celite, macro-porous anion exchange resin, silica, macro-porous acrylic resin and polyurethane as carriers. Studies proved that FAEE yield is higher in the reaction catalyzed by immobilized enzyme compared to that of freely suspended enzyme (Shah et al. 2004).

The use of lower alcohols like methanol and ethanol is restricted in enzyme catalyst trans-esterification process because it can deactivate the immobilized enzymes. From conventional alcohol ethanol is more preferred in this due to lower enzyme deactivation value. Propan-2-ol, ethyl acetate or diethyl carbonate as acyl acceptors are also used in the biodiesel production from non-edible oils to overcome enzyme inactivation. The step wise addition of methanol and organic solvents can also be used. The ester yield is not affected in the presence and absence of an organic solvent but it increases the rate of reaction considerably. The immobilized enzyme can

be used several times to reduce the production cost making them stable using propan-2-ol and ethyl acetate. No loss in enzymes activity has been seen because of better solubility of propanol-2-ol and ethyl acetate in TAG and less polarity than chain alcohols.

In enzyme catalyzed reaction water plays an important role by increasing the stability and catalytic activity of an enzyme and hence, contributing in higher ester yield. Contrary, water also have role in ester hydrolysis which decreases the ester yield (De Oliveira et al. 2004). Many studies with *Candida antractica* (De Oliveira et al. 2004) proved that the higher ester yield is achieved in absence of water.

Production of biodiesel using enzymes is mainly done using batch laboratory reactors but one-step packed bed reactor has been reported without substrate loss over 500 h. This result is very important for practical application of biodiesel production in-spite of the disadvantage that solvent recovery is not possible.

9.3.4 Supercritical Trans-Esterification Process

The process of trans-esterification of plant oils under supercritical conditions using alcohols has been considered as the most important process for biodiesel production. For researchers it is the most promising process to be used at large scale in future. In this process, the lag-phase of the reaction which is caused by the low solubility of alcohols in the oil phase can be overcome giving high rate of reaction with shorter period of time. It simplifies the separation and purification of biodiesel with no soap formation in saponification and no waste is generated and glycerol recovery is easier as there is no use of catalyst. This process is having advantages at every step of biodiesel production but the main disadvantage of using this process degradation of the esters formed at very high temperature and the increased cost of the reactors to withstand high temperature and pressure. Absence of catalyst in the reaction requires higher alcohol and oil molar ratio to start the trans-esterification reaction and for complete conversion of oil into esters. The ester yield increased by increasing the molar ratio of alcohol and oil because of increased contact area between TAG and alcohol. This process is best suitable for plant oils containing low content of FFA. Similar results are obtained using supercritical methanol and ethanol. However, the supercritical methanol is having higher conversion degree than supercritical ethanol (Demirbas 2009).

Studies have also done to see the effect of temperature and pressure on supercritical trans-esterification and it was found that increase in temperature increases the ester yield independently of the raw material used (Demirbas 2009). The temperature higher than the critical temperature of methanol and ethanol is used which is in between 240 °C and 244 °C. the complete TAG conversion into esters can be achieved at a pressure of 20 MPa and increase in the reaction pressure increases the yield. The reason behind the increased yield is the probably the effect due to the increase of the density with increasing the pressure which in turn increases the solvent power of the supercritical fluid (Campanelli et al. 2010). Some studies also shows that the conversion under supercritical conditions takes less time as compared to the conventional methods using catalysts (Campanelli et al. 2010).

Some scientists have also applied a two-step process as a better alternative to the one-step supercritical method. It involves the hydrolysis of TAG molecules in subcritical water at 270 °C and the supercritical trans-esterification and separated fatty acids without catalysts in supercritical methanol. Using this two-step method increases the FAME yield compared to conventional methods described before and also a valuable by product glyoxal is formed instead of glycerol and is not influenced by high FFA content. One can also use dimethyl carbonate in place of methanol but dimethyl is costlier than methanol or ethanol.

9.4 Biodiesel Properties of Plant Oil

The biodiesel produced from plant oils should meet the standard biodiesel specifications to be used in engines giving better efficiency as set by the ASTM International. The standard specifications issued by ASTM International are generally comparable to THE European standard EN 14214 and the National Standard of Canada CAN/CGSB-3.524. The specification standard also specifies different test methods to be used in the determination of properties for biodiesel blends. To be used as a commercial fuel, the finished biodiesel has to undergo certain sophisticated techniques to ensure its specifications as per ASTM even if it has to be stored for longer times.

9.4.1 Properties of Biodiesel from Edible Oil

The physical and chemical properties of biodiesel produced from edible oils with properties of crude oils are listed below (Tables 9.1 and 9.2)

Table 9.1 Physical and chemical properties of crude oils of some edible plant oils [35]

S.No.	Property	Coconut	Palm	Canola	Soybean	Moringa
1	Kinematic viscosity (mm^2/s) at 40 °C	27.640	41.932	35.706	31.7390	43.4680
2	Kinematic viscosity (mm^2/s) at 100 °C	5.9404	8.496	8.5180	7.6295	9.0256
3	Dynamic viscosity (mpa s) at 40 °C	25.123	37.731	32.286	28.796	38.9970
4	Viscosity index (VI)	168.5	185.0	213.5	223.2	195.20
5	Flash point (°C)	264.5	254.5	290.5	280.5	263
6	CFPP (°C)	22	23	15	13	18
7	Density (kg/m^3) at 40 °C	0.9089	0.8998	0.9042	0.9073	0.8971
8	High calorific value (kJ/kg)	37,806	39,867	39,751	39,579	39,762
9	Copper strip corrosion (3 h at 50 °C)	1a	1a	1a	1a	1a
10	Refractive index	1.4545	1.4642	1.471	1.4725	1.4661
11	Transmission (%T)	91.2	63.2	62.9	65.2	69.2
12	Absorbance (Abs)	0.04	0.199	0.202	0.186	0.16
13	Oxidation stability (h at 110 °C)	6.93	0.08	5.64	6.09	41.75

9.4.2 Properties of Biodiesel from Non-edible Oil

The physical and chemical properties of biodiesel produced from non-edible oils with properties of crude oils are listed below (Tables 9.3 and 9.4)

Table 9.2 Physical and chemical properties of the biodiesel produced from edible oils [35]

S.No.	Property	Coconut	Palm	Canola	Soybean	Moringa
1	Viscosity (mm^2/s) at 40 °C	3.1435	4.6889	4.5281	4.3745	5.0735
2	Viscosity (mm^2/s) at 100 °C	1.3116	1.7921	1.7864	1.764	1.9108
3	Viscosity (mpa s) at 40 °C	2.705	4.0284	3.9212	3.8014	4.3618
4	Density (kg/m^3) at 40 °C	0.8605	0.8591	0.866	0.869	0.8597
5	Oxidation stability (h at 110 °C)	8.01	23.56	7.08	4.08	12.64
6	CFPP (°C)	−1	12	−10	−3	18
7	Cloud point (°C)	1	13	−3	1	21
8	Pour point (°C)	−4	15	−9	1	19
9	Flash point (°C)	118.5	214.5	186.5	202.5	176
10	Copper strip corrosion (3 h at 50 °C)	1a	1a	1a	1a	1a
11	High calorific value (kJ/kg)	38,300	40,009	40,195	39,976	40,115
12	CCR (m/m%)	0.0114	0.0118	0.0291	0.0204	0022
13	Total sulfur (mg/kg)	0.94	1.81	0.83	0.86	–
14	Absorbance (Abs)	0.035	0.05	0.041	0.037	0.046
15	Transmission (%T)	92.3	89.1	91.1	92	90
16	Refractive index	1.4357	1.4468	1.4544	1.4553	1.4494
17	Viscosity index (VI)	230.8	203.6	236.9	257.8	206.7

Table 9.3 Physical and chemical properties of crude oils of some non-edible plant oils [35]

S.No.	Property	Jatropha	Sterculia	Calophyllum	Croton	Patchouli
1	Kinematic viscosity (mm^2/s) at 40 °C	48.095	75.826	55.478	29.8440	9.8175
2	Kinematic viscosity (mm^2/s) at 100 °C	9.1039	13.608	9.5608	7.2891	2.2151
3	Dynamic viscosity (mpa s) at 40 °C	43.543	69.408	51.311	27.1570	9.2933
4	Viscosity index (VI)	174.1	184.8	165.4	224.20	−21.60
5	Flash point (°C)	258.5	246.5	236.5	235	146.5
6	CFPP (°C)	21	29	26	10	1
7	Density (kg/m^3) at 40 °C	0.9054	0.9153	0.9249	0.9100	0.9466
8	High calorific value (kJ/kg)	38,961	39,793	38,511	39,331	42,986
9	Copper strip corrosion (3 h at 50 °C)	1a	1a	1a	1a	1a
10	Refractive index	1.4652	1.4651	1.4784	1.4741	1.5069
11	Transmission (%T)	61.8	26.6	34.7	87.5	71.4
12	Absorbance (Abs)	0.209	0.574	0.46	0.058	0.146
13	Oxidation stability (h at 110 °C)	0.32	0.15	0.23	0.14	0.14

Table 9.4 Physical and chemical properties of the biodiesel produced from non-edible oils [35]

S.No.	Property	Jatropha	Sterculia	Calophyllum	Croton	Patchouli
1	Kinematic viscosity (mm^2/s) at 40 °C	4.9476	6.3717	5.5377	4.0707	6.0567
2	Kinematic viscosity (mm^2/s) at 100 °C	1.8557	2.1954	1.998	1.6781	1.8223
3	Dynamic viscosity (mpa s) at 40 °C	4.2758	5.5916	4.8599	3.453	5.5848
4	Density (kg/m^3) at 40 °C	0.8642	0.8776	0.8776	0.8704	0.9221
5	Oxidation stability (h at 110 °C)	4.84	1.46	6.12	0.71	0.022
6	CFPP (°C)	10	2	11	−4	−17
7	Cloud point (°C)	10	1	12	−3	< − 33
8	Pour point (°C)	10	2	13	−2	< − 33
9	Flash point (°C)	186.5	130.5	162.5	164	118.5
10	Copper strip corrosion (3 h at 50 °C)	1a	1a	1a	1a	1a
11	High calorific value (kJ/kg)	39,738	40,001	39,513	39,786	44,180
12	CCR (m/m%)	0.0440	0.2911	0.4069	0.028	0.385
13	Total sulfur (mg/kg)	3.84	7.02	4.11	–	77.1
14	Absorbance (Abs)	0.045	0.057	0.057	0.041	3
15	Transmission (%T)	90.3	87.9	87.7	91.1	0
16	Refractive index	1.4513	1.4557	1.4574	1.4569	1.5032
17	Viscosity index (VI)	194.6	236.9	183.2	276.3	61.8

9.5 Challenges of Biodiesel Production from Plant Oil

The twin crisis of depleting fossil fuel and global warming has necessitated the search of other transportation fuel as an alternative to fossil fuel. Biodiesel emerged as a potential fuel in recent years. Currently, the Europe and US are the leaders of biodiesel market. In 2005, the total consumption of biodiesel was 3.32 million tons/year. However, this value was only 3% of total fuel usage in Europe and US. European society has also committed to increase the biofuel usage to 20% by 2020. Therefore, the demand for biodiesel is going to increase sharply worldwide.

In Indian context, transportation sector required almost 70% of total fuel consumption and this demand is increasing day by day. Thus, in India, use of biodiesel is much more important than in any other country. Apart from being high in demand, biodiesel is facing lots of challenges such as social and economical, technical and environmental issues with its use and production. Various scientific journals as well as media highlighted the problems associated with biodiesel production and its use and these are:

(a) Competition of plants for food and fuel
(b) Levels of greenhouse gases
(c) Deforestation and soil-erosion
(d) Loss of biodiversity of land as well as water
(e) Modifications required in fossil fuel engines
(f) Production cost

To meet the demands of biodiesel, an in depth study of all direct and indirect factors are required to enhance the biodiesel production by eliminating the issues with proper solution.

9.5.1 Land Crises Issue

The first and fore most challenge in the biodiesel production is the availability of land to grow the raw material at large scale. The estimates for land requirements for future biodiesel production vary widely and depend on the type of feedstock, geographical locations and the percentage of yield. Large area of land is needed to grow edible plants for food supply and non-edible plants to meet the fuel supply. And there the competition arises for land to be used for plants for food supply or for biodiesel production. Food being the basic requirement of living beings needs more land and hence, the land crises issue arises for the plants to be used in biodiesel production. Most of us are aware about the debate on "Food vs. Fuel" on many modes of media communication.

The availability of land to be used to raise the oil plants for biodiesel is very limited. In some parts of the world the forests are converted into farm lands for growing feedstock required as a raw material in biodiesel production. Growing of plants at large scale is a preliminary step for the process of biodiesel production to be completed but manufacturers are facing problems with land. The use of forests for growing the feedstock leads to deforestation, soil erosion and many more negative impacts. Due to which the first step of biodiesel production is a continuous topic of discussion in market. The use of wasteland can significantly reduce the land crises issue. Even hybrid crops having high oil content can be used to give high yield in less land available in coming future.

9.5.2 Impact on Food Supply

Eager to enhance the use of renewable fuels to minimize dependency on fossil fuels in the country. The Brazil, The United States and The European Union are promoting the use of biodiesel made from edible as well as non-edible oils. To reduce the oil insecurity of the country by converting oil seeds into biodiesel and other biofuels is generating global food insecurity.

As per the report by HLPE on food security and nutrition in June 2013, explain the increase in food price and food insecurity due to increase in biofuel production. Hence, the reason of debate and controversy are still very active within the research and scientific community. Different geographical conditions and their impact on food transport. The use of biodiesel is generally in developed nations and is affecting the food security in food insecure countries by transmission of high international prices in local markets. IUFoST states that by 2030, the global population will increase by 17% and so world food production must increase by 50% to satisfy the needs. There is a major concern that the increasing demand for energy and

expansion of biodiesel crop production. This expansion will threaten the food security of poor sectors of the world in near future by sharp hike in prices in international food market.

The conversion of farmlands to raise oil plants for biodiesel and high price offers given to farmers for their lands by biodiesel producers is creating food insecurity even at the small scale. The food security can be maintained by using the lands that are not suitable for crop production and by growing non-edible plant for biodiesel production.

9.5.3 Impact on Environment

The increasing dependency on biofuels in developed countries has a direct impact on the land and environment. To reduce the emission of greenhouse gases and effects of global warming produced by fossil fuels is one of the main reasons for producing biofuels. However, according to FAO of the UN, the effects of biodiesel are also seen on land, water and biodiversity. They are affected by agricultural production and if the agriculture production is intensified then side effects are even greater. Majority of the people are having conception that growing of crops for biodiesel production reduces the greenhouse gases as they directly remove carbon dioxide present in the air.

The biodiesel as well as fossil diesel release the same amount of carbon dioxide but plants raised for biodiesel production absorb atmospheric carbon dioxide. On the other side the use of nitrous oxide in fertilisers that are put on the ground to help the crops grow will have 300 times more global warming effect than carbon dioxide. The conversion of forest land to grow the plants for biodiesel production is responsible for deforestation, soil erosion and loss of biodiversity. The intensification of crop production through new technology and impacts on soil through water use and potential decline of water level could be limiting factor in biodiesel production. A.N. Shah and his group in 2013 also focused on the emissions of carbonyl compounds like formaldehyde, acetaldehyde, acrolein, acetone, propionaldehyde and butyraldehyde are higher in biodiesel mixtures than emissions from pure diesel but lower total hydrocarbon emissions.

9.6 Future Work and Prospects

Decline in fossil fuels and increasing demand for fuels will automatically increase the production of biofuels significantly in the near future. The growing need for biodiesel production justifies the need to find economical and sustainable feedstock. The data presented in the above sections clearly defines that edible as well as non-edible plant oils can be used for biodiesel production. Economically, palm oil is the cheapest oil in the world would be the most suitable for biodiesel production.

Advancement in research and need of biodiesel is going to have a boom in the production of biodiesel. Edible as well as non-edible plant oils are going to raise their prices due to increase in demand.

9.7 Conclusions

DVC Process Technologists offers the Conclusion best solutions for commercial viability in the processing of the most challenging feed stocks, through both chemicals as well as enzymatic routes over various capacities. The biodiesel production usually involves the following steps:

1. **Feedstock Pre-treatment**: Most Feedstocks used in Biodiesel production are of low grade and contain impurities that reduce the catalyst efficiency in transesterification and hence have to be pretreated.
2. **Transesterification and/or Acid Esterification**: This is the main reaction in biodiesel production which involves the conversion of the triglycerides and/or fatty acids into methyl esters. The conversion costs depend on yields, feedstock quality, choice of catalyst (Sodium Methoxide, Sulphuric acid or Enzyme), process routes, energy consumptions, etc.
3. **Glycerine Recovery**: The glycerine obtained from biodiesel production must be further purified, concentrated & distilled to get technical grade or pharmaceutical grade of glycerine.
4. **Methanol Recovery**: The excess methanol from the various streams is recovered to be re-used again for the transesterification process.
5. **Biodiesel Purification**: Biodiesel Purification involves washing of the methyl ester phase and drying. For higher quality of biodiesel, methyl ester distillation may also be necessary.

9.8 Summary

In coming few years the demand for biodiesel is expected to hike sharply round the globe because of a decline in fossil fuels and their out of the pocket prices. Solution to problems related to environmental pollution and its negative impacts on human health. Hence, increase in the biodiesel production using edible as well as non-edible plant oils. But the problem arises in choosing the feedstock for biodiesel production. Using the edible oils for biodiesel production due to the competition of edible oils as a source of food containing essential fatty acids is a topic of discussion for researchers across the world for biodiesel production. So, other feedstocks like non-edible oils and animal fats are taking place of edible plant oils place in biofuel production industries. The increasing demand of biodiesel is a highly concerned topic for the scientists to look into the methods of production with low by product and waste production and high biodiesel production by reducing the overall cost of production and production time. Trans-esterification is a series of reaction behind

the complete conversion of fatty acids into esters for higher yield of biodiesel. Studies are continuously trying to give better methods for production at large scale with minimal side effects. The use of solid catalysts instead of homogenous one is desirable due to its reduced environmental properties and higher biodiesel production. However, a complete heterogeneous method is not yet known. The continuous urge to develop and use of mixed catalysts (acid/base) to decrease the cost by using two-step process leads to switching on enzymes and supercritical methods of production. These processes reduce the production cost considerably by using no catalyst and reusability of enzymes in enzyme catalyzed method of production but still the cost of enzyme is very high and is the main drawback of enzyme catalyzed method. The alcohol quantity, reaction time, reaction temperature and catalyst concentration are main factors affecting the yield of biodiesel. Different methods of separation and purification are also there, washing using hollow fiber membrane extraction seems promising because it avoids emulsification and also decreases the refining loss. The biodiesel produced by the methods described in the chapter is eco-friendly in nature but before using this product biodiesel in the engines it needs to meet the standard biodiesel specifications set by the ASTM International for better efficiency.

In spite of having many advantages over the use of fossil fuel the biodiesel production is facing many challenges. The first and fore most challenge in the production is the availability of land to grow the raw material at large scale. Large area of land is needed to grow edible plants as edible plant oil contains valuable nutrients which can't be ignored and are beneficial to human health and are therefore more worthy to be used as food supplement rather than to be used in engines. On the other hand even large area is required to grow non-edible plants for biodiesel production. And there the competition arises for land to be used for plants for food supply or for biodiesel production. Hence, it is a major topic seeking the solution by examining all the positive and negative sides of both the issues impacting human life. Therefore, the feedstock for biodiesel production should come from diversified sources depending on geographical conditions. Proper land management is required to keep the balance between agriculture land food supply plants and wasteland for non-edible plants. This management will ensure that different feedstocks can be used for biodiesel production. Growing of crops for biodiesel is contributing to large scale deforestation of mature trees, loss of habitat, reduced biodiversity and imbalance of greenhouse gases leading to global warming. Still the use of biodiesel is advised over fossil fuel by examining the data available in literature about the amount of greenhouse gas emissions from biodiesel combustion and many studies reveal that biodiesel contribute very less to greenhouse gases due to complete combustion of the fuel. Ultimately, biodiesel can be a sustainable resource replacing the fossil diesel without affecting the food market globally.

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Effect of Nanomaterials and Their Possible Implication on the Plants

10

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Abstract

Nanomaterials have found extensive applications in a wide array of life and industrial processes. The potential uses and benefits of nanotechnology in agriculture are important because the production of food increases with minimum cost and energy. Recently, the researcher has implemented nanomaterials in agriculture for the improvement of the crop yields. Nanomaterials could increase or decrease crop growth as well as yield. However, they can also migrate to the food chain with implications for humans and animals. In this book chapter, the effects of different nanomaterials on plant growth, intake and bioaccumulation/biotransformation and toxicological risks for food materials have been discussed. The chapter also addresses recent aspects regarding nanomaterials and the environment, with an emphasis on the plants.

Keywords

Nanomaterials · Agricultural sectors · Engineered nanomaterials · Crop plants

10.1 Introduction

Nanotechnology has been worldwide used in different sector like the medical field, agri-food industry, pharmaceutical sector, etc. (López-Moreno et al. 2010; Ong et al. 2018; Thiruvengadam et al. 2018). Although nanomaterials (NMs) present in the environment, due to the increasing demand of nanotechnology application in

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our daily life, engineered nanomaterials (ENMs) production should be increased for the fulfillment of demand.

Many researchers proved that the increased the ion concentrations in the plant due to the environmental conditions (Kinnersley and Scott 2001; Isla and Aragüés 2010). Under specific growing environments, an excess concentration of elements either essential or nonessential uptake by plants after requirements, caused toxicity (Ke et al. 2007). In addition, excess elements in plant tissues caused fatal effects on nontolerant species (Castillo-Michel et al. 2009; Arias et al. 2010). The excess element either beneficial or toxic in plants can be transmitted to consumers from primary producers. For example, selenium-accumulated plants can be used to provide ruminants and other animals with selenium deficiencies (Whanger 2002; Hefnawy and Tórtora-Pérez 2010); however, it is still being examined due to the limits between toxicity and deficiency are very narrow (Zhu et al. 2009).

Some of the nanomaterials are known to be reactive and toxic to plants either directly or indirectly by releasing ions, altering bacterial species present in the soil, damage to the roots and the absorption of co-contaminants by plants (Ma et al. 2011; Ge et al. 2014). Thus, Nanomaterials have caused the increase or decrease of crops growth or yield and can also enter the food chain and affect the humans and animals (Shaw et al. 2014; Thiruvengadam et al. 2015; Xiang et al. 2015; Vannini et al. 2013).

10.2 Effects of the Different Nanomaterials on Plants

Natural nanomaterials (NNMs) and engineered nanomaterials (ENMs) are present in the environment where plants or crop species may interact with them. Generally, carbon nanomaterials, metal oxide and metallic nanoparticles can affect the plant species.

10.2.1 Carbon Nanomaterials

Carbon nanomaterials have found applications in optical devices, conducting materials, molecular switching, biological contaminant removal, tissue engineering, pharmaceutical sector, and drug delivery system (Upadhyayula et al. 2009; Kostarelos et al. 2007; Ali-Boucetta et al. 2011; Serag et al. 2013). The production of fruit crop and vegetables affected by the carbon nanomaterials. The use of carbon nanomaterials in agriculture is a fundamental need due to the growing population and the depletion of resources. CNTs are the key to the growth of plants and fruits because of the penetration of CNTs into the plant. The penetration of CNTs is totally dependent upon size and concentration. Plant cell do not take CNTs easily, due to their bigger size. Therefore, they get adsorbed on the surface (Khodakovskaya et al. 2012). The carbon nanomaterial has been revealed to increase water retention capacity, which in recent years has been a remarkable achievement in nanotechnology. The tomato production was increased for about 200% by using 50 μg/ml carbon nanotubes (Husen and Siddiqi 2014).

MWCNTs have no huge impact after 5 days of treatment on seed germination of corn, cucumber, lettuce, radish, and rape at the concentration of 2 g/l (Lin and Xing 2007). Similarly, no phytotoxic side effects or increased physiological reactions were accounted for on living wheat roots (Wild and Jones 2009). At 10–40 mg/l concentrations, MWCNTs increased the germination percentage of tomato seed and vegetative mass in relation to the control. This could be due to the increased water consumption caused by the CNTs (Khodakovskaya et al. 2009). On the other side, the high concentration of SWCNTs and their functionalized form were present on the bottom of the apical meristem of tomato roots affected root elongation (Canas et al. 2008). The reproduction of rice plants was inhibited by C70 and MWCNT at a concentration of 400 mg/l for at least 1 month (Lin et al. 2009a, b). Interestingly, exposure to MWCNT has also led to self-protection and oversensitive reaction in rice cells (Tan and Fugetsu 2007; Tan et al. 2009).

10.2.2 Metal Oxide Nanomaterials

In the agriculture sector, many kinds of metal oxide nanoparticles (NPs) like Fe_3O_4, CeO_2, TiO_2, and ZnO have been applied for the plant protection and fertilization and also study for the toxicity (Gogos et al. 2012). Some metal oxide nanoparticles like TiO_2 NPs could significantly encourage the plant growth (Hong et al. 2005); and when treated with ZnO, the root length of the green peas was about two times longer than the control (Mukherjee et al. 2014). TiO_2 nanoparticles reduced the cell pore diameter, due to its accumulation in root cell walls of maize plant caused the phytotoxicity in crop plants (Asli and Neumann 2009). Mixtures of silica and titanium oxide nanoparticles enhanced the activity of nitrate reductase, stimulated the antioxidant system in soybean and improved water and fertilizer absorption (Lu et al. 2002). TiO_2 NPs in spinach was reported to enhance the formation of chlorophyll and dry weight of plants (Zheng et al. 2005; Hong et al. 2005). For instance, Some researcher reported that TiO_2 could extensively reduce the seed germination and elongation of maize root (Castiglione et al. 2011), while some of the researchers also showed that TiO_2 had no major effects (Burke et al. 2014).

ZnO NPs at high concentrations do not appear to affect the germination of seeds, but experimental data showed that soybean root elongation was effected by lower concentration (López-Moreno et al. 2010a, b). Also, ZnO NPs were associated with highly vacuolated cortical cells and collapsed as vascular cells decreased and died partially (Lin and Xing 2008). Copper and zinc oxide could considerably reduce the cucumber growth by 44%, respectively (Kim et al. 2013). The effect of ZnO NPs mediated phytotoxicity and other interactions on agriculturally important microbe like *Azotobacter* was studied for wheat and tomato seeds (Boddupalli et al. 2017).

In contrast to ZnO NPs, nanoceria reduced seed germination in high concentrations of alfalfa, soybean, tomato, and cucumber (López-Moreno et al. 2010a, b). However, at the minimum concentration, corn germination was significantly reduced. ZnO and CeO_2 NPs was toxic to the Corn at low concentration and did not harmful for other crops (Lin and Xing 2007; López-Moreno et al. 2010a, b). The surface properties of the nanoparticles cause the toxicity. The metal oxide

nanoparticles were found to enhance the dry weight of soy pod and leaf (Sheykhbaglou et al. 2010).

10.2.3 Metallic Nanomaterials

Plants are directly exposed to the naturally available metallic nanomaterials in the environment. The germination frequency is increase by silver nanoparticles, however, the extent of enhancement depends on size, shape, and concentration. The gold nanoparticles, on the other hand, inhibit the seed germination (El-Temsah and Joner 2012). Zucchini seeds (*C. pepo*) germination was completely inhibited by Si nanoparticle when it was present with sodium dodecyl sulfate (used as surfactant make to suspension), while 80% germination resulted in the absence of SDS (Stampoulis et al. 2009).

10.3 Uptake and Transformation of Nanomaterials into Plant

The uptake of nanomaterials either carbon-based or metal-based by plants is the latest study area. Current literature has shown that the absorption, translocation, and storage of nanomaterials rely upon the plant species and nature of the nanomaterials. In contrast, most metal-based nanoparticles have been absorbed and stored in plants. The absorption, biotransformation, and translocation of different nanoparticles by a model plant was shown in Fig. 10.1. Several studies have shown that plants

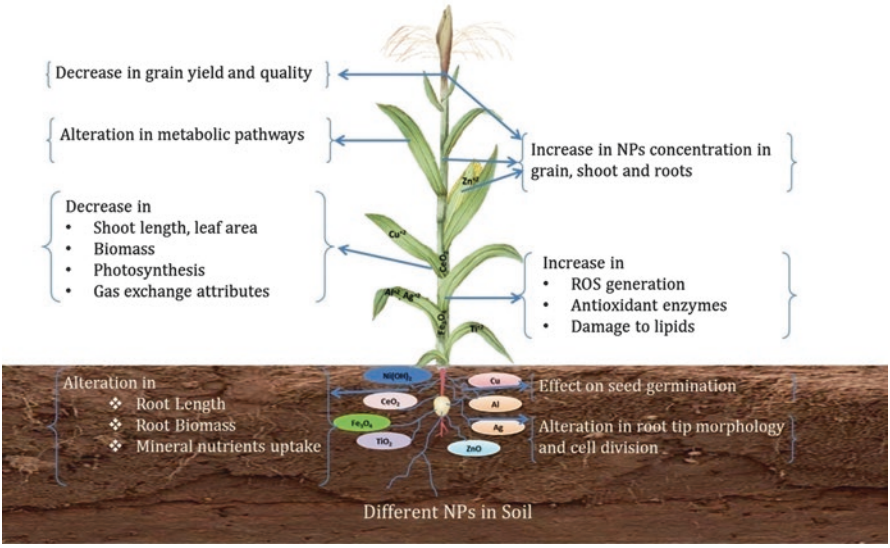

Fig. 10.1 Schematic diagram of uptake, biotransformation, and translocation of various nanoparticles

can absorb nanoparticles through the soils (Du et al. 2011; Zhao et al. 2012a, b; Shi et al. 2014). Nanoparticles can adhere to root surfaces and enter via an apoplastic pathway through plant epidermis and cortex (Kurepa et al. 2010; Du et al. 2011; Rui et al. 2014; Zhao et al. 2012a, b; Zhu et al. 2008; Zhou et al. 2011; Zhao et al. 2012a, b). However, uptake and translocation of nanoparticles in plants differ with different plant species (Servin et al. 2012; Slomberg and Schoenfisch 2012; Zhao et al. 2013a, b; Li et al. 2014).

10.3.1 Uptake of Carbon-Based Nanomaterials

Carbon nanotubes (CNTs) are used as supply systems for different biomolecules/genes/drugs into the cells, and investigation are carried out to determine the absorption and transportation mechanisms of CNTs into plant cells. Khodakovskaya et al. reported that MWCNTs were used for the development of tomato seedlings by seeds and root systems. (Khodakovskaya et al. 2009). It was assumed that the MWCNTs could enter through small pores of seed coat and improve water absorption. Also, it was seen on the root surface and pierced into the cell walls (Wild and Jones 2009). The hydrophobic characteristics of the MWCNT allow them to interact with many organic substances (Yang et al. 2006). This property was investigated for herbal remediation purposes. C_{60} increased the absorption of trichloroethylene by almost 80% in cotton cuttings at 15 mg/l (Ma and Wang 2010). Some researcher reported that the entry of the MWCNTs into the cytoplasm was restricted by cell walls of rice cell (Tan and Fugetsu 2007). It was also reported that MWCNTs form black clumps that closely wrap around and relate to the cells (Tan et al. 2009). The numbers and sizes of the clumps were increased with increasing concentration. The absorption of nanomaterials in plants natural ecosystems is likely to be dependent on the chemical properties of the soil. The presence of black aggregates C_{70} in the seeds and roots was more abundant than the other part of rice (Lin et al. 2009). Therefore, the carbon-based nanomaterials can be taken up by plants and they provoke the programmed cell death and also some times enhanced the growth of the plant. In order to found the mechanisms of their accumulation in the edible plant, further studies are required.

10.3.2 Uptake of Metal Oxide Nanomaterials

Titanium oxide nanoparticles are used extensively in day to day life, and the study of their absorption and relocation in plants is limited. TiO_2 nanoparticles complexed with Alizarin red S was relocated into the tissue of *A. thaliana*, because the roots of *A. thaliana* developed pectin hydrogel capsule around itself due to the released of mucilage, which help to promote the entry of Alizarin red S or sucrose-complex TiO_2 (Kurepa et al. 2010).

The absorption, relocation, and storage of zinc oxide nanoparticles are not well understood in plants crops. Additionally, most studied were conducted up to the germination stage, providing limited information due to the incomplete development of the plant root (López-Moreno et al. 2010a, b). Zinc nanoparticle at 0.5 g/l concentration have low aggregation and have higher absorption by the seedlings, while at higher concentrations, there are chances for the formation of aggregation and resulted to reducing intake and storage of nanoparticles. The pumpkin seedlings uptake of Fe_3O_4 nanoparticles in hydroponic conditions was studied and found that the signal for magnetic NPs was detected in different parts of pumpkin plants (Zhu et al. 2008). Cerium has been accumulated in soybean, maize and tomato tissues with increased external concentrations of CeO_2 NPs (López-Moreno et al. 2010a, b). The difference in the accumulation of nanoparticles in plants due to the root microstructures differences, their physiochemical interactions and the presence of root exudates in the rhizosphere. CeO_2 NPs were absorbed by maize leaves but could not be transferred to new leaves (Birbaum et al. 2010).

10.3.3 Uptake of Metallic Nanomaterials

The absorption of Ag NPs in zucchini is 4.7 times higher than in their corresponding bulk counterpart (Stampoulis et al. 2009). It was suggested that more release of ions from Ag NPs is accountable for the higher concentration of Ag in shots. *Brassica juncea* plants treated with silver nanoparticles did not appear to store silver in any way in another study (Haverkamp and Marshall 2009). The absorption and translocation of copper nanoparticles in mungbean and wheat in agar growth medium has been investigated and they have crossed the cell membrane (Lee et al. 2008). Biotransformation and accumulation of Ag(I) and Pt(II) ions into Pt and Ag NPs have also been reported in alfalfa and mustard seedlings (Bali et al. 2010; Harris and Bali 2008). It can be concluded that plant uptake the elemental ion and bioaccumulate in the form of nanoparticles. These nanoparticles can increase or decrease the growth of the root and shoot.

10.4 Toxic Effect of Nanomaterials on Plants

Nanoparticles caused either positive/no consequential effects (Table 10.1) or negative/toxic effect (Table 10.2) in plants. Nanoparticles 'toxicity depends on their chemical composition and the release of toxic ions or on the stress caused by the size and shape of nanoparticles (Brunner et al. 2006). The various factor of nanoparticles like concentration and particle, specific surface area, physicochemical properties, stability influences toxicity in food crops (Keller et al. 2010). Nanoparticles toxicity could be caused by plant species and growth media (Keller et al. 2010; Lin et al. 2009). The size of the seeds could make them more sensitive to exposure to nanoparticles (Canas et al. 2008; El-Temsah and Joner 2012).

Table 10.1 Positive effects of nanoparticles on plants

Nanoparticles	Plants	Toxic effect	References
Zero-valent Fe	Flax, Red clover, White clover, Meadow fescue	No effect on germination	El-Temsah and Joner (2012)
Zero-valent Fe	Barley, Ryegrass	No effect on germination	El-Temsah and Joner (2012)
Zero-valent Fe	Flax, Barley, Ryegrass	Completely inhibited germination	El-Temsah and Joner (2012)
Zero-valent Fe	Barley	Reduced germination	El-Temsah and Joner (2012)
Zero-valent Fe	Flax, Barley, Ryegrass	No germination	El-Temsah and Joner (2012)
Al	*Raphanus sativus*, *Brassica napus*	Improved root growth	Lin and Xing (2007)
Au	*Cucumis sativus*, *Lactuca sativa*	Positive effect on germination index	Barrena et al. (2009)
CeO_2	*Cucumis sativus*, *Zea mays*	Increased root and stem growth	López-Moreno et al. (2010a, b)
CeO_2	*Coriandrum sativum* L.	Increased shoot and root length, biomass, catalase activity in shoots and ascorbate peroxidise activity in roots	Morales et al. (2013)
TiO_2	*Triticum aestivum* L. *var.* Pishtaz	Increased shoot and seedling lengths	Feizi et al. (2012)
Ag	*Trigonella foenum-graecum* L.	Enhanced plant growth and diosgenin synthesis	Jasim et al. (2017)
ZnO	*Arachis hypogaea*	Improved growth and yield	Prasad et al. (2012)
ZnO	*Cyamopsis tetragonoloba*L.	Improved shoot-root	Raliya and Tarafdar (2013)
		Growth, chlorophyll, total soluble leaf protein content, rhizospheric microbial population and P nutrient-mobilizing enzymes including phytase, acid and alkaline phosphatise	
SiO_2	*Lycopersicum esculentum*Mill	Improved seed germination	Siddiqui and Al-Whaibi (2014)
Single-walled carbon nanotube	Onion, Cucumber	Significantly increased root length	Canas et al. (2008)
Single-walled carbon nanotube	Cabbage, Carrot, Lettuce	No effect	Canas et al. (2008)
Functionalized single walled	Cabbage, Carrot, Tomato, Onion, Lettuce	No effect	Canas et al. (2008)
Carbon nanotube			

(continued)

Table 10.1 (continued)

Nanoparticles	Plants	Toxic effect	References
Multiwalled carbon nanotube	Tomato	Significant increase in germination rate, fresh biomass, and length of stem	Khodakovskaya et al. (2009)
		Significantly enhanced moisture content inside	
		Tomato seeds	
Multiwalled carbon nanotube	Radish, Rape seed, Ryegrass, Lettuce, Corn, Cucumber	No effect on germination	Lin and Xing (2007)
Multiwalled carbon nanotube	Ryegrass	Increased root length	Lin and Xing (2007)
Multiwalled carbon nanotube	Zucchini	No effect on the germination	Stampoulis et al. (2009)
Multiwalled carbon nanotube	Wheat	No significant effect on root or shoot growth	Wild and Jones, (2009)

Table 10.2 Negative effect of nanoparticles on plants

Nanoparticles	Plants	Toxic effect	References
Zero-valent Fe	Flax, Barley, Ryegrass	Completely inhibited germination	El-Temsah and Joner (2012)
	Barley	Reduced germination	El-Temsah and Joner (2012)
	Flax, Barley, Ryegrass	No germination	El-Temsah and Joner (2012)
Ag	Triticum aestivum L.	Reduced shoot and root length	Dimkpa et al. (2013)
Ag	Hordeum vulgare L.	Reduced germination	El-Temsah and Joner (2012)
Ag	*Allium cepa*	Decreased mitosis, disturbed metaphase, sticky chromosome, cell wall disintegration and breaks	Kumari et al. (2009)
Ag	*Linum usitatissimum* L., cv. Electra, *Lolium perenne* L., cv. Tove	Reduced shoot length	El-Temsah and Joner (2012)
Ag	*Cucurbita pepo*	Reduced transpiration	Stampoulis et al. (2009)
Zn	*Zea mays*, *Cucumis Sativus*, *Lactuca sativa*, *Raphanus sativus*, *Brassica napus*, *Lolium perenne*	Reduced root growth and elongation	Lin and Xing (2007)
Cu	*Phaseolus radiatus*	Reduced seedling growth	Lee et al. (2008)
Cu	*Cucurbita pepo*	Reduced biomass and root growth	Stampoulis et al. (2009)

(continued)

Table 10.2 (continued)

Nanoparticles	Plants	Toxic effect	References
Al_2O_3	*Zea mays*, *Cucumis sativus*, *Brassica oleracea*, *Daucus carota*	Reduced root growth	Yang and Watts (2005)
Al_2O_3	*Zea mays*	Reduced root length	Lin and Xing (2007)
CeO_2	*Lycopersicon esculentum*	Reduced shoot growth	López-Moreno et al. (2010a, b)
CeO_2	*Brassica nigra*	Reduced growth due to high oxidative stress	Sakla et al. (2016)
CeO_2	*Zea mays*	Reduced shoot growth	López-Moreno et al. (2010a, b)
ZnO	*Zea mays*	Reduced germination	Lin and Xing (2007)
TiO_2	*Anabaena variabilis*	Inhibition in cell growth and nitrogen fixation activity	Cherchi and Gu (2010)
TiO_2	*Triticum aestivum*L. *var.* Pishtaz	Reduced germination	Feizi et al. (2012)
Al	*Lolium perenne*	Decreased root length	Lin and Xing (2007)
Al	*Lolium perenne*	Reduced germination	Lin and Xing (2007)
Al	*Zea mays*, *Lactuca sativa*	Reduced root length	Lin and Xing (2007)
Single-walled carbon nanotube	Rice	Delayed flowering, decreased yield	Lin et al. (2009)
Single-walled carbon nanotube	Tomato	Most sensitive in root reduction	Canas et al. (2008)
Functionalized carbon nanotube	Lettuce	Reduced root length at longer exposure	Canas et al. (2008)
Multiwalled carbon nanotube	Zucchini	Reduced biomass (38%)	Stampoulis et al. (2009)
Multiwalled carbon nanotube	Lettuce	Reduced root length	Lin and Xing (2007)
Multiwalled carbon nanotube	Rice	Chromatin condensed inside the cytoplasm and caused cell death, plasma membrane detachment from cell wall and cell shrinkage	Tan et al. (2009)

10.4.1 Effect of Nanomaterials on Plant Physiological Indices

The toxic effects of nanomaterials on plant physiological indices are root/shoot elongation, germination percentage and increase/decrease in biomass (Lee et al. 2012). The negative side effects of nanomaterials on plants are decrease in seed germination, inhibition of plant growth, and sometimes also caused plant death. Nanomaterials have a toxic effect on plant growth including seed germination and shoot length (Dimkpa et al. 2012; El-Temsah and Joner 2012). *Bacillus thuringiensis* (Bt)-transgenic cotton was treated with the SiO_2 nanoparticles, growth was inhibited (Le Van et al. 2014). CuO nanoparticles inhibit the growth of wheat plants and also affect the root structure (Dimkpa et al. 2012; Tang et al. 2016). CuO nanoparticles also affect the germination of rice seed and reduced the root length of *Arabidopsis* seedlings (Shaw and Hossain 2013). Carbon nano-dots exposure with plants leads to improved wheat plant growth as compared to controls (Tripathi and Sarkar 2015). Effects of 10–11 days of exposure to multi-wall carbon nanotubes at different concentrations on soybean, maize and barley germination in agar medium (Lahiani et al. 2013). In comparison with untreated controls, almost 50% increase in germination rates in Hordeum vulgare and Glycine max and 90% in Zea mays was observed during exposure. In Glycine max, the root lengths increased to 26% and in Zea mays; shooting and leaf lengths increased by 40% and more than three-fold (Lahiani et al. 2013). Toxicity of carbon nano-materials was found to be largely dependent on their concentrations, growth/ exposure conditions, and plant species. Therefore, the toxic effect of nanomaterials to plants are observed like reduction in seed germination, inhibition of plant elongation, and sometimes also caused plant death. Also sometimes the positive effect of nanomaterial on the plant are observed in terms of an increase in germination and growth rate.

10.4.2 Effect of Nanomaterials on Plant Hormones and Seed Germination

Plant metabolism produces active organic materials called plant hormones. Effect of nanoparticles on plant hormones was studied elaborately because it regulate physiological responses of plant growth (Santner et al. 2009). Nanoparticles had different effect on different plant hormones. For example, CeO_2 nanoparticles had no significant effect on abscisic acid (ABA), indole-3-acetic acid(IAA) and gibberellic acid (GA) in the leaves of cotton compared with the control (Le Van et al. 2015). Whereas, when the transgenic and non-transgenic rice was expose with iron oxide nanoparticles, an increase in ABA and IAA content in the roots was observed (Gui et al. 2015).

Many researchers reported the toxic effects of metal nanoparticles on seed germination of because it is the primary step to determines the crop growth in nanomaterials contaminated soils (Thiruvengadam et al. 2015; Moon et al. 2014; Lin and Xing 2007; Mandeh et al. 2012). In general, the seed germination rate of many crops species can be affected by the toxicity of nanoparticles (Wu et al. 2014; Rizwan et al. 2017). The effect of silver nanoparticles on seed germination of some

plants like rice, barley, turnip, and faba bean was studied (El-Temsah and Joner 2012; Thuesombat et al. 2014; Thiruvengadam et al. 2015). Some plant like turnip, barley and faba bean was shown decreased in seed germination growth as compared to the control by silver nanoparticles. Similarly, reduced seed germination was obtained, when the plant species treated with metal oxide NPs (CuO, NiO, TiO_2 and Fe_2O_3 nanoparticles) because nanoparticles absorbed on seed surface and release metal ions (Wu et al. 2014). The seed germination and size was varied with the crops (i.e., lettuce > cucumber > radish). Different soils caused the variation in seed germination (El-Temsah and Joner 2012). The silver nanoparticles and silver ion present in the soil to enhanced the seed germination rate of sorghum (Lee et al. 2012). Similarly, the silver nanoparticles present in soil caused less inhibited of seed germination of radish and lettuce (Gruyer et al. 2013). Copper oxide nanoparticle at the concentration at 600 mg/ml lowered the germination rate and also the rice seed germination was reduced in a dose-dependent manner of nanoparticles (Moon et al. 2014; Shaw and Hossain 2013). In general, the nanoparticles regulate the plant hormone either positive and negative way in species specific manner.

10.4.3 Effect of Nanomaterials on Grain Yield and Quality

The overall food crops was effected and reduced by the toxicity of nanoparticles. The effects of CeO_2 NPs on quality and quantity of crops differ with plants species. For example, CeO_2 nanoparticles presence at 500 ppm in soil, increased yield and quality of wheat (An et al. 2008; Rico et al. 2015; Peralta-Videa et al. 2014). In another study, the same concentration of CeO_2 nanoparticles (500 ppm in soil) was applied with barley, The seed was not formed and observed opposite results (Rico et al. 2015). The cucumber yield was decreased by 31.6% with the concentration of 800 mg/kg of CeO_2 nanoparticles (Zhao et al. 2013a, b) because this nanoparticle altered the quality of carbohydrates, mineral nutrients and proteins of cucumbers (Zhao et al. 2014). CeO_2 and ZnO nanoparticles reduced the maize yield respectively by 38% and 49% (Zhao et al. 2015). Also, similar results were observed in soybean plants exposed with CeO_2 and ZnO nanoparticles (Peralta-Videa et al. 2014). In addition, CeO_2 nanoparticles decreased the Mo concentration in cucumber fruits and altered phenolic content, non-reducing sugars, and fractionation of proteins (Zhao et al. 2014). Titanium oxide nanoparticles reduced the Potassium and phosphorus in cucumber fruits (Servin et al. 2012). Silver nanoparticles increased the potassium (K) content while reduced phosphorus (P), magnesium (Mg), and Sulphur (S) in tomato fruits (Antisari et al. 2015).

10.5 Summary and Conclusion

The plants are affected directly or indirectly by the nanomaterials present in the environment. Evidently, the exposure and assimilation of nanoparticles did affect the growth, photosynthetic efficiency, quality and yield of grains. However, the precise toxic effects of nanoparticles on grain/fruits development and quality of

crop product would permit more indepth investigation. Different nanomaterials (Carbon-based, metallic oxide and metallic nanomaterials) have varied effect in genera specific manner. It could be toxic and inhibitory towards one plants genera, while could enhance the seed germination and root/shoot growth as well as grain yield towards others. In the future, these nanomaterials can be used as nanopesticides for crop protection or as an agent for improvement in crop yield by tailoring the type, size, and shape of nanomaterials.

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Generating Bioelectricity from Different Organic Residues Using Microbial Fuel Cells

11

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Abstract

Nowadays with an increase in industrialisation there is also an increase in the need of such techniques that can protect the environment and living being from the harmful effects of the industrialisation. The techniques may involve the use of natural resources like wind, sunlight or tidal or may include the use of waste materials like food wastes, waste water. The use of waste to give us fuel or electricity is one of the recent discoveries that do not harm the environment and fulfil the needs. One of such technique is the microbial fuel cell (MFC), it is a bioelectric system used for generating bioelectricity with the help of waste materials. MFCs consist of an anode chamber, cathode chamber and a separating membrane. It makes use of microorganisms that act as biocatalysts in converting the waste materials into electricity. The good power output and efficient conductivity differs with the use of material in making the electrodes and separating membrane plus the quality of waste material used as substrate. In this chapter the detailed overview of the structure and function of anode and cathode chamber, separating membrane are given along with the different type of waste used for producing bioelectricity.

Keywords

Waste materials · Microbial fuel cell · Bio-electricity

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11.1 Introduction

In this era of industrialization and globalization the increasing demand of energy is being fulfilled with the help of non-renewable resources, renewable resources and nuclear resources. Among all these three sources of energy only renewable sources are beneficial as the other two sources of energy produce good amount of energy in different form but along with it cause danger to the environment by causing pollution. In such a situation several renewable resources to complete the energy demand have been discovered like solar energy, wind energy, tidal energy etc. Among all these a new approach of fuel cell has also been found that has emerged as one of the most reliable source of energy production in last few years.

In 1911, Potter observed electricity production with the help of bacteria (Potter 1911) and around 1990 fuel cells were worked upon leading to the discovery of microbial fuel cells that emerged as one of the most reliable source of bioelectricity production. Microbial fuel cells are bio-electrochemical systems (BES) that are capable of converting biomass into electricity with the help of metabolic activity of microorganisms used. Microbial fuel cells consist of anode chamber and cathode chamber that are separated by proton exchange membrane (PEM) that is ion-selective membrane (Ghassemi and Slaughter 2017). Microorganisms are used as catalysts that help in the production of protons and electrons by oxidizing carbon sources and organic substrates present in the anode chamber (Kumar et al. 2018). Protons from the anode chamber are passed on to the cathode chamber via proton exchange membrane and the electrons produced are passed to an external circuit (Rahimnejad et al. 2011a, b). Protons and electrons react in the cathode chamber where parallel reduction of oxygen to water takes place side by side (Sharma and Li 2010). In the anode chamber the microorganisms are kept in anaerobic condition as the presence of oxygen in this chamber would disturb the electricity production (Jiang et al. 2017). Proton exchange membrane prevents any oxygen from entering the anode chamber and helps in the charge transfer between anode and cathode. In anode chamber the growth of microorganisms take place and in the cathode chamber electrons produced by the anode chamber react with oxygen.

11.2 Structure and Function of Anode Chamber

Anode chamber is one of the main parts of microbial fuel cell and it is maintained in anaerobic condition. Its function is to degrade the organic matter used as substrate and generate electricity. It is filled with substrate that can be any organic waste matter rich in carbon, microorganisms, anode electrode that generates electrons and protons. Bacteria are mostly used catalytic microorganisms that degrade the organic material used as substrate (Yuniati 2018), anodic microbial electron transfer, material of anode, configuration of electrode influence the effectiveness of a microbial fuel cell (Ulcer et al. 2017). Electrodes are essential parts as they amplify the electron transfer rate and should have the following properties: strong biocompatibility,

good electric conductivity and low resistance, large surface area, anti-corrosion and chemical stability.

The materials used in anode are carbon containing materials like: carbon paper, graphite rod, reticulate vitreous carbon (RVC), graphite fiber brush and carbon felt. All these materials have good microbial stability, large surface area and good electric conductivity. Along with these materials granular activated carbon (GVC) and graphite granules (GG) can also be used as they possess micro- porosity, good conductivity and are affordable. Recently researchers are modifying anode with nano engineering techniques as this would improve the electron transfer and conductivity. Anode made of carbon nano tube (CNT) and polyaniline nanostructure is found to amplify electro transfer and increase surface area. Polyaniline is mostly used to modify anode increasing its microbial stability and polyaniline combined with fluorine, carbon nano-tubule or titanium dioxide has resulted in good current outputs. Polytetrafluroethylene (PTFE) is also used in anode modifications as it is hydrophobic and has chemical stability. PFTE combined with graphite having 30% (w/w) PFTE and *Escherichia coli* used as biocatalyst resulted in power density 760 mW/m^2 (Zhang et al. 2007). Table 11.1 showed different types of MFC.

Table 11.1 Different MFC and the anode used

Anode	Microorganism	Power density (mW/m^2)	References
Carbon paper	*G. sulfurreducens*	48.4 ± 0.3	
Graphite	*Saccharomyces cerevisiae*	16	
Carbon paper	*Geobacter* sp. (Firmicutes)	40.3 ± 3.9	
Noncorroding graphite	*Desulfurmonas* sp.	25.4_26.6	
Platinum and polyanilineco-modified	*Escherichia coli*	6000	
Teflon treated carbon fiber paper	Electrochemically active bacteria	15.2	Rahimnejad et al. (2011a, b)
Graphite	*Deltaproteo* bacterium	14	
Non-wet-prof carbon paper	Cellulose degrading bacteria	188	Logan et al. (2006)
Carbon paper with PPYCNTs	*Escherichia coli*	228	
Composite electrode (graphite/PTFE)	*Escherichia coli*	760	Rahimnejad et al. (2011a, b)
Carbon paper	*Geobacter* sp.	52 ± 4.7	
Graphite with Mn^{4+}	*Escherichia coli*	91	Park and Zeikus (2002)
Carbon paper	*Gammaproteo* and *shewanella affinis* (KMM3586)	36	
Graphite with neutral red (NR)	*Escherichia coli*	152	Park and Zeikus (2002)

11.3 Structure and Function of Cathode Chamber

After degradation of organic matter anode compartment produces electrons and protons that are passed into the cathode chamber via proton exchange membrane (PEM) thus completing an electrical circuit. The electrons produced convert the oxygen into water in the cathode chamber and current is produced by this reaction. The total current produced by the cathode depends on nature of the oxidant (electron acceptor), availability of protons and the structure of cathode and its catalytic efficiency. Oxygen is mostly used as an electron acceptor as it has intense oxidation potential, is easily accessible and produces no waste products (Ulcer et al. 2017). To enhance MFCs performance alternative oxidants have been discovered like potassium permanganate and very less amount of it can increase the power output (Allami et al. 2018). Biocathodes are new alternatives of normal cathodes in which microorganisms are used for catalyzing cathodic reactions and yielding good power density (Angioni et al. 2018). They do not use any artificial mediator or catalyst as electron acceptor and remove any byproducts formed by microbial metabolism. The structure, design and surface are of cathode affects the efficiency of MFCs. Cathode can be made from different materials like carbon brush, carbon paper, carbon fiber, carbon felt, graphite, platinum, tungsten carbide, copper, Cu-Au, graphite granules, reticulated vitreous carbon (RVC) (Chen et al. 2008). Different materials used in making cathode give different energy outputs as given in the Table 11.2.

Table 11.2 Different materials used in making cathode

Cathode	Maximum current density	Maximum power density	Maximum voltage	References
Air–cathode with graphite	1210 mA/m^2	283 mW/m^2	440 mV	Rahimnejad et al. (2011a, b)
Tubular ACFF	4.69 mA/m^2	0.3 W/m^3	644 mV	Deng et al. (2009)
Biocathode	3.34 A/m^2	667 W/m^3	658 mV	Chen et al. (2008)
Activated carbon fiber felt (ACFF)	1.67 × 10^{-3} mA/m^2	315 mW/m^2 (0.7 W/m^3)	679 mV	Deng et al. (2009)
Plain carbon	1.5 mA/m^2	67 mW/m^2 (0.1 W/m^3)	598 mV	Deng et al. (2009)
Graphite felt	3145 mA/m^2	539 mW/m^2	742.3 mV	
Air–cathode with Carbon cloth	363 A/m^3	50 W/m^3	710 mV	You et al. (2008)
ACFF granules	3.34 A/m^2	667 W/m^3	658 mV	Deng et al. (2009)
Plain carbon	1.5 mA/m^2	67 mW/m^2 (0.1 W/m^3)	598 mV	Deng et al. (2009)
Parallel sheets of carbon paper secured by carbon fiber coated with Pt	13.16 A/m^3	7.29 W/m^3	553 mV	Fornero et al. (2008)
Carbon felt	6 × 10^{-3} mA/m^2	77 mW/m^2 (0.2 W/m^3)	575 mV	Deng et al. (2009)

11.4 Structure and Function of Membrane

Proton exchange membrane is often referred to separator that separates anode chamber from the cathode chamber as different reactions take place in both of them. They are one of the main components of MFCs as they allow transfer of protons but prevent any transfer of substrates and oxygen. There is increased pH in anode chamber and decreased in cathode chamber, this is called pH splitting. This pH splitting results in increased internal resistance, decreased system performance, and stability (Watanabe 2008). To improve the efficiency of separators they were made by using materials like ion exchange membranes, salt bridge, size specific membranes (Li et al. 2011). Ion exchange membrane can be classified into anion exchange and cation exchange membrane on the basis of the type of ion they separated and they consisted of bipolar membranes, ultrafilteration membranes, glass fibers, porous fabrics (Zhuang et al. 2009), salt bridge.

Different types of exchange membranes used in MFCs are as follows:

11.4.1 Cation Exchange Membrane (CEM)

It is an ion-penetrable membrane that transfers positively charged ions and are also known as proton exchange membranes. Cations offer less internal resistance and high conductivity to the MFCs because of which they are mostly used (Moruno et al. 2018; Sun et al. 2009a, b). Different materials used for making CEM are: dialysed membrane, ultrex, divinylbenzene with sulphuric acid, nafion, nano-porous filters, bipolar membranes, microfilteration membrane and glass wool (Rahimnejad et al. 2012). Among all these Nafion is commonly used, it was first developed by Dupont in 1970 (Ghasemi et al. 2013). Nafion is a perfluorosulphuric acid membrane having backbone of hydrophobic fluorocarbons ($–CF^2–CF^2–$) on which negatively charged hydrophilic sulphonated groups ($SO–^3$) are present because of which Nafion has high conductivity to different cations. Nafion enhance the cell performance as they have appropriate thickness and level of hydration but they cannot work in neutral pH and in presence of certain cationic species like Na+, K+ because they increases the pH in cathode chamber. The major disadvantage of using it is that it increases the cost of the MFCs and is physically unstable at high temperatures (Zawodzinski et al. 1995).

Ultrex is another reliable material used in CEM as it is affordable and physically stable at higher temperatures, one of its examples is Ultrex CMI 7000. CMI 7000 is a membrane of strong acid polymer having divinylbenzene and gel polystyrene cross-linking and having sulphonic acid groups attached. Zirfon (Pant et al. 2010) and Hyflon are other alternative materials used for CEM. Zirfon consists of 15% of polysulfone, a macroporous mineral material and 85 wt% of hydrophilic ZrO2

(Vermeiren et al. 2008). It has high oxygen permeability that is not good for anodic reactions but it has low internal resistance (Vermeiren et al. 2008), good conductivity and physical stability compared to Nafion. Hyflon is also effective as compared to Nafion but it has high internal resistance. Nowadays researchers are finding affordable and effective nanofiber and nanoparticle membranes. Recently a membrane having Fe_3O_4/PES nanocomposite with Fe_3O_4 and *Saccharomyces cerevisiae* used as biocatalyst showed high conductivity in a MFC (Rahimnejad et al. 2012). It has been found that most of the CEM have certain disadvantages like high internal resistance, pH splitting, high oxygen penetration, low proton transfer and huge costs.

11.4.2 Anion Exchange Membrane (AEM)

It uses pH buffers of phosphates and carbonates that enhance the transfer of protons (Zhuang et al. 2012). In this type of membranes substrate permeability is higher than CEM and it makes use of positive ions like SR^{2+}, NH^{3+} that help in the transfer of anions. A cathode having AEM based membrane resulted in 13.1 W/m^3 of power density where CEM resulted only in 8.3 W/m^3.

11.4.3 Bipolar Membranes (BPM)

It consists of two monopolar membranes that facilitate in the transfer of protons and hydroxide ions improving electrical conductivity, reducing cost and weight of a MFC (Zhuang et al. 2012; Dihrab et al. 2009). Metals like graphite and stainless steel are used for making this membrane. Graphite is fabricated because of which it has better conductivity but less power output is being produced. To improve stainless steel is used along with it as it improves the power density and chemical stability (Dihrab et al. 2009).

11.5 Different Substrates Used

The production of electricity in a MFC depends upon the structure and nature of the organic matter used and the concentration and chemical composition of the components that are degraded. Substrate influences the bacterial biocatalyst used, power density and columbic efficiency of MFCs. Table 11.3 showed different types of substrates used for MFC production and Table 11.4 gives of different types of MF cells.

Table 11.3 Different substrates used for MFC production

Substrate used	Inoculums	Current density(mA/cm^2)	References
Azo dye with glucose	Mixture of aerobic and anaerobic sludge	0.09	
Acetate	Pre-acclimated bacteria from MFC	0.8	
Carboxymethyl cellulose (CMC)	Co-culture of *Clostridium cellulolyticum* and *G. sulfurreducens*	0.05	
Arabitol	Pre-acclimated bacteria from MFC	0.68	Catal et al. (2008a, b)
Cysteine	Two-chambered MFC with carbon paper as electrodes	0.0186	Logan et al. (2005)
Ethanol	Two-chambered aqueous cathode MFC with carbon paper electrodes	0.025	Kim et al. (2007)
Corn stover biomass	Domestic wastewater	0.15	Zuo et al. (2006)
Furfural	Pre-acclimated bacteria from anode of a ferricyanide-cathode MFC	0.17	
1,2-Dichloroethane	Microbial consortia from acetate enriched MFC	0.008	Pham et al. (2009)
Farm manure	Self build up of anaerobic environment	0.004	Scott and Murano (2007)
Cellulose particles	Pure culture of *Enterobacter cloacae*	0.02	Rezaei et al. (2009)
Lactate	Pure culture of *S. oneidensis* MR-1	0.005	Manohar and Mansfeld (2009)
Glucose	Mixed bacterial culture maintained on sodium acetate for 1 year (*Rhodococcus* and *Paracoccus*)	0.70	Catal et al. (2008a, b)
Macroalgae, *Ulva lactuca*	Primary clarifier overflow of wastewater plant	0.25	Velasquez-Orta et al. (2009)
Galactitol	Pre-acclimated bacteria from MFC	0.78	Catal et al. (2008a, b)
Landfill leachate	Leachate and sludge	0.0004	Greenman et al. (2009)
Microalage, Chlorella vulgaris	Primary clarifier overflow of wastewater plant	0.20	Velasquez-Orta et al. (2009)
Malt extract, yeast extract and glucose	Pure culture of *E. cloacae*	0.067	Mohan et al. (2008a, b)
Phenol	Mixed aerobic activated sludge and anaerobic sludge (1:1, v/v)	0.1	

(continued)

Table 11.3 (continued)

Substrate used	Inoculums	Current density(mA/cm^2)	References
Mannitol	Pre-acclimated bacteria from MFC	0.58	Catal et al. (2008a, b)
Sodium formate	Anaerobic digested fluid from a sewage treatment plant	0.22	
Propionate	Anaerobic sludge	0.035	Oh and Logan (2005)
Nitrilotriacetic acid (NTA)	Oligotrophic consortium enriched with river water	0.0005	Jang et al. (2006)
Ribitol	Pre-acclimated bacteria from MFC	0.73	Catal et al. (2008a, b)
Microcrystalline cellulose	Rumen microorganism from rumen of a cow	0.02	Rismani-Yazdi et al. (2007)
Sucrose	Anaerobic sludge from septic tank	0.19	Behera and Ghangrekar (2009)
Sorbitol	Pre-acclimated bacteria from MFC	0.25	Catal et al. (2008a, b)
Xylitol	Pre-acclimated bacteria from MFC	0.71	Catal et al. (2008a, b)
Starch	Pure culture of *Clostridium butyricum*	1.3	Niessen et al. (2004a, b)
Sodium fumarate	Pure culture of *G. sulfurreducens*	2.05	Dumas et al. (2008)
Brewery wastewater	Full strength brewery wastewater	0.2	Feng et al. (2008)
Xylose and humic acid	Domestic wastewater	0.06	Huang et al. (2008)
Chocolate industry wastewater	Activated sludge	0.302	Patil et al. (2009)
Xylose	Mixed bacterial culture	0.74	Catal et al. (2008a, b)
Beer brewery wastewater	Anaerobic mixed consortia	0.18	Wen et al. (2009)
Meat processing wastewater	Domestic wastewater	0.115	
Domestic wastewater	Anaerobic sludge	0.06	Wang et al. (2009)
Protein-rich wastewater	Mesophilic anaerobic sludge	0.008	Liu et al. (2009)
Starch processing wastewater	Starch processing wastewater	0.09	Lu et al. (2009)
Real urban wastewater	Domestic wastewater	0.18	Rodrigo et al. (2007)
Food processing wastewater	Anaerobic sludge	0.05	Oh and Logan (2005)

(continued)

Table 11.3 (continued)

Substrate used	Inoculums	Current density(mA/cm^2)	References
Swine wastewater	Full-strength swine wastewater	0.015	Min et al. (2005)
Paper recycling wastewater	Diluted paper recycling wastewater	0.25	Huang et al. (2008)
Synthetic wastewater	Anaerobic mixed consortia producing hydrogen	0.086	
Wastewater amended with acetate	Domestic wastewater	0.08	Min and Angelidaki (2008)
Synthetic wastewater	Anaerobic culture from a preexisting MFC	0.017	Aldrovandi et al. (2009)

11.6 Different Types of MFC (Table 11.4)

Table 11.4 Different microbial fuels cells

1. Mediated Fuel Cell	In these types of fuel cells the electron transfer from microbial cells to electrode is facilitated by different mediators like methyl viologen, humic acid, methyl blue, thionine. They are electrochemically inactive.
2. Mediator free	In these fuel cells bacteria is used for transfer of electrons to the electrode. The most common electrochemically active bacteria are Aeromonas hydrophila, Shewanella putrefaciens some bacteria transfer the electron via pilli on their surface. These fuels cells can work only with waste water and can also produce energy directly from some plants. Such fuels cells are called plant microbial fuel cells, they use plants like lupines, algae, rice, tomato, reed sweetgrass, cordgrass etc.
3. Microbial electrolysis	It is a variation of mediator free microbial fuel cell. As normal microbial fuel cells produce electricity by decomposition of bacterial compounds in water, but microbial electrolysis cells apply voltage to bacterial cells that reverses the process generation hydrogen or methane. This reverse in process supplements the voltage generated by microbial decomposition of organics leading to electrolysis of water or production of methane.
4. Soil based	Here soil acts as nutrient rich anodic media, proton transfer membrane and inoculums. In these cells anode is inserted at a particular depth within the soil, and the cathode remains in the top soil interacting with air.
	Soil is a good source of several electrogenic bacteria and has complex sugar and nutrient depositions from dead and decaying plants and animals. The aerobic microbes present in the soil act as filters for oxygen causing redox potential of the soil to decrease with depth.
5. Phototrophic biofilm	They use phototrophic biofilm anode that contain photosynthetic microorganisms. They carry out photosynthesis and produce electrons. When they use complete oxygenic photosynthetic material they are called biological photovoltaic cells.
6. Nonporous membrane	They use non permeable electron membrane to generate passive diffusion within the cells. The membrane used is a non porous filter like nylon.
7. Ceramic membranes	Here the PEM is replaced by ceramic membrane as the macroporous structure of ceramic membranes allows efficient transport of ionic species.

11.7 Advantages and Disadvantages of Using MFC

The greatest benefit of using MFC is that it can utilise the useless waste water and organic matter. The waste water and organic matter can be mechanically or chemically treated as per the requirement. They can be used to derive energy from useless sources. Such strategy has two ecological impacts first can be stated as the organic waste can be utilized directly in these fuels which used to be mechanically or chemically treated otherwise, in this process the energy expenditure is reduced for the treatment. Secondly, it has a lower impact as in greenhouse gas emission than some other fuel cells as this process does not rely on fossil fuels. Because of their usage the waste water treatment facilities can become self sufficient or can even produce high amount of electricity as these fuel cells can be coupled with wastewater. So there has been a great interest in using microbial fuel cells in municipal wastewater treatment. As we know bacteria are less expensive and can be grown easily which can be used to produce fuel cells and then the use of MFCs avoid the use of expensive catalyst such as platinum. Overall MFCs are environment friendly, have easy installation, low maintenance and simpler working mechanism.

Focusing on the disadvantages, they can produce limited power. Microbial fuel cells have found application in powering devices that are remote monitoring and have low power needs as they produce low power density. It is costly, has less life, and some elements in the cell have rare availability and some other technical difficulties due to which it is still in development phase.

11.8 Future Prospects

Microbial fuel cells are one of the emerging fields in energy production using waste water and waste organic compounds. They have been studied for their working, microbial interactions, configuration, electrochemistry etc. it easily converts the low grade waste water into electricity but the amount of electricity produced is not very good and the process is costly. So for better production research is being going on for increasing the amount of electricity produced and to decrease the cost.

11.9 Conclusion

This overview of MFC, structure and function of different chambers showed how the material of electrodes can show different current outputs. The importance of separating membrane for the proper working of MFC has also being explained. The substrates along with different microorganisms used to catalyse the reaction producing electricity are mentioned along with the power output and the difference with different substrate can be noticed.

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Progress and Prospects in the Production of Cellulosic Ethanol

12

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Abstract

Bioethanol as a biofuel has received significant attention in the recent years. This is expected to provide a solution to the heavy reliance on petroleum based products for energy security, adverse effect on the environment and climate change due to emissions of vehicles. India, being an agriculture-based economy, produces huge quantities of agricultural residues after harvesting the crops. This chapter will, therefore, focus on the availability of agro-residues in India, the assessment of pretreatments, saccharification processes such as acidic and enzymatic methods and fermentation of hydrolysates. The progress achieved in the use of various wild and mutant strains of microbes and recombinants for the production and development of cellulose-hydrolyzing enzyme cocktails and their utility in the saccharification of agro-residues will be discussed. The role of microbial strains in fermenting cellulosic hydrolysates, the need for genetic engineering approach and consolidated bioprocess development for the production of bioethanol will be critically reviewed.

Keywords

Agro-residues · Cellulosic hydrolysates · Alcoholic fermentation · Cellulolytic enzymes · Bioethanol · Thermostable enzymes · Recombinant enzymes

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12.1 Introduction

The increasing costs and green house effects caused by fossil fuels are creating a core demand for exploring alternative cheaper, eco-friendly and renewable bio-fuels (Asgher et al. 2013; Iqbal et al. 2013). A wide variety of lignocellulosic biomass resources are available as low-cost potential candidates, which can be converted to bioethanol. In the past few decades, a considerable progress has been made in utilizing lignocellulosic biomass for bioethanol production. The lignocellulosic biomass contains carbohydrates, which are first converted into simple sugars (e.g. glucose) with the help of cellulolytic enzymes and the sugar produced is fermented to ethanol (Alonso et al. 2008; Balat and Balat 2009; Lin and Tanaka 2006) by ethanologenic microbial strains. The conversion of lignocellulosic biomass to biofuel requires a three-step process that includes: (i) pre-treatment (mechanical, chemical, or biological), (ii) enzymatic hydrolysis and (iii) fermentation of sugars in cellulosic hydrolysates (Wyman 1999; Xiao et al. 2012). The third largest industrial enzymes worldwide are cellulases, and the interest in cellulases has grown because of their utility in the production of bioethanol as well as in food processing, paper recycling, cotton processing and detergent industries. Fungi and bacteria have been exploited, to produce a wide variety of cellulases. The emphasis has been on the production of cellulases from various microbial sources, and cloning and expression of cellulase encoding genes in *E.coli, Pichia pastoris* and filamentous fungi.

Extensive efforts are being made to produce significant quantities of cellulosic ethanol. Recently, several attempts have been made worldwide to produce bioethanol in pilot scale plants. The chapter deals with the strategies developed in selecting most appropriate lignocellulose pretreatment methods, production of cellulolytic enzymes and fermentation operations. There are too many challenges in terms of robustness, hydrolysis efficiency and cost effectiveness of cellulolytic enzymes. Despite substantial progress made in this field in the last two decades, extensive research efforts are called for overcoming the problems and limitations encountered in developing a viable bioprocess.

12.2 Structure and Composition of Lignocellulosic Biomass

Lignocellulosic biomass is composed of cellulose and hemicellulose (~70%) which are tightly associated to the lignin component via covalent and hydrogen bonds. This bonding makes the structure highly robust and resistant to any treatment (Limayem and Ricke 2012). Lignocellulosic biomass is the most abundant renewable and cheap source of energy, with a yearly production of ~200 × 10^9 tons (Zhang 2008). Typical composition of various lignocellulosic residues is listed in Table 12.1. There are many lignocellulosic agricultural residues that are available for bioethanol production such as sugarcane bagasse, cereal straws, corn cobs, switch grass, softwood and several others. In agriculture dominated countries like India, the crop residues offer a great potential for ethanol production. The availability of potential

lignocellulosic crop residues in India and their potential ethanol yields are summarized in Tables 12.2 and 12.3, respectively.

Table 12.1 Composition of lignocellulosic biomass (% of dry matter)

Lignocellulosic residue	Cellulose	Hemicellulose	Lignin	References
Paddy straw	28–36	23–28	12–14	Han and Rowell (1997)
Wheat straw	33–38	26–32	17–19	Han and Rowell (1997)
Barley straw	31–45	27–38	14–19	Han and Rowell (1997)
Sorghum straw	15	12.3	5.8	Billa et al. (1997)
Sugarcane bagasse	32–48	19–24	23–32	Han and Rowell (1997)
Corn stover	38–40	28	17–21	Reddy and Yang (2004)
Corn cobs	38.8	44.4	11.9	Pointner et al. (2014)
Corn stalks	29.8	33.3	16.65	Shawkya et al. (2011)
Coconut coir	36–43	0.15–0.25	13	Majumdar and Chanda (2001)
Miscanthus	41.9	26.6	13.3	Magid et al. (2004)
Switchgrass	46.1	32.2	12.3	Reshamwala et al. (1995)
Oat straw	37.60	23.34	12.85	Adapa et al. (2009)
Canola straw	42.39	16.41	14.15	Adapa et al. (2009)

Table 12.2 Gross and surplus crop residue biomass potential in India (Hiloidhari et al. 2014)

Crop	Gross Potential, MT[a]	Surplus Potential, MT
Rice	154	43.5
Wheat	131.1	28.4
Sugarcane	110	55.7
Maize	35.8	9
Barley	1.6	0.2
Sorghum	17.6	3.5
Millet	24.3	5.1
Coconut	18	9.7
Jute	3.9	0.4
Groundnut	17	3

MT [a] metric ton

Table 12.3 Bioethanol production potential using agro-residues (Sukumaran et al. 2017)

Crop residues	Ethanol yield (L/ton)
Rice straw	209.0
Rice husk	198.14
Sugarcane bagasse	244.97
Wheat straw	242.29
Corn cob	258.65
Corn stover	231.90
Corn husk	201.0
Sorghum stover	230.0

12.2.1 Cellulose

Cellulose is present in plant cell walls in the range of approximately 35–50% of plant dry weight and is the most abundant component of plant biomass. The cellulose fibres are entrenched in the matrix of other structural biopolymers, mainly hemicelluloses and lignin, which comprise 20–35% and 5–30%, respectively. Cellulose is crystalline and amorphous in structure and a natural high molecular weight polymer composed of a long-chain of D-glucopyranose units linked by β-1, 4 glycosidic bonds that can range several thousand glucose units in length. The extensive hydrogen bonds in the molecule lead to a crystalline and a strong matrix structure. Individual cellulose molecules are assembled into larger units called protofibrils, which are packed into larger units known as microfibrils, these in turn are assembled into cellulose fibers.

12.2.2 Hemicellulose

Hemicellulose is the second most abundant branched heteropolymer of D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid. The backbone chain is primarily composed of D-xylose (~90%) and L-arabinose (~10%) via β-1,4 linkages. Branch frequencies vary depending on the nature and the source of feedstocks. The soft wood hemicelluloses are usually glucomannans, while hardwood hemicelluloses are more commonly composed of xylans. Because of the diversity of its sugars, hemicellulose requires a wide range of enzymes for its hydrolysis into monomers.

12.2.3 Lignin

Lignin is usually the most complex fraction that accounts for about 10–25% of the biomass by weight. It is an aromatic and rigid biopolymer composed of three phenolic monomers of phenyl propanoid alcohols (coumaryl, coniferyl and sinapyl alcohols) linked by ether bonds. Lignin comprises complex and large polymer of phenylpropane, methoxy groups and non-carbohydrate polyphenolic substance that binds cell wall components together (Hamelinck et al. 2005). It provides structural support and resistance to oxidative stress and microbial attack. The composition of lignin also varies with the nature of feedstocks. Forest woody biomass is mainly composed of cellulose and lignin polymers. The highest amount of lignin (30–60%) is present in softwood barks followed by hardwood barks (30–55%), whereas low proportions of lignin 10–30% and 3–15% are present in grasses and agricultural residues, respectively (Demirbas 1998; Pettersen 1984).

12.3 Pretreatment Methods

The conversion of biomass to ethanol requires steps such as pretreatment of biomass, enzymatic hydrolysis and fermentation and product recovery. The appropriate combination of each step is significant in order to achieve high bioethanol yields in a cost-effective and sustainable manner. The structural complexity of lignocellulosic biomass strongly restricts the cellulase accessibility to cellulose to liberate fermentable sugars. Therefore, efficient pretreatment of biomass is required in order to make the enzymes accessible to cellulose. The main challenge in ethanol production from lignocellulosic biomass is the pretreatment. The pretreatment process breaks the matrix of cellulose and lignin bound by hemicelluloses, thus reducing the cellulose crystallinity. The pretreatment converts the recalcitrant lignocellulosic structure to reactive cellulosic intermediates before enzymatic hydrolysis. Cellulose hydrolysis yields increased to 90% after pretreatment, which was only 20% in untreated biomass (Lynd 1996).

12.3.1 Physical Pretreatments

The objective of physical pretreatment is to increase surface area, decrease degree of polymerization, decrystallization and reduce particle size of lignocellulosic materials. The physical methods include milling, grinding, chipping, freezing and radiation. One of the important features of physical pretreatments is that the production of toxic materials is negligible. In physical pretreatment, particle size is reduced by chipping, milling or grinding that increases surface to volume ratio. Even irradiation (γ-rays) is also known to lower crystallinity and provide large surface area by cleavage of β-1,4-glycosidic bonds (Kumar et al. 2009). Pyrolysis has also been used as the pretreatment method. In this process, biomass is treated at temperatures greater than 300 °C that leads to decomposition to gaseous products and residual char (Kumar et al. 2009). Attempts have also been made on microwave pretreatment method (Zhu et al. 2006; Binod et al. 2012; Choudhary et al. 2012). In this method, the biomass is irradiated with microwaves for a short time period, which is effective in removing lignin and hemicellulose components from the biomass. This method offers several advantages over conventional heating processes, such as short processing time and low energy demand. It has been found that microwave pretreatment is more effective when used with other chemical pretreatment methods.

Factors such as capital costs, operating costs and scale-up possibilities are very important for physical pretreatment. Silva et al. (2010) studied the effect of ball milled sugarcane bagasse and straw; glucose and xylose yields were 78.7%, 72.1% and 77.6%, 56.8%, respectively. Ethanol yield from total fermentable sugars was 89.8% and 91.8% from bagasse and straw hydrolysates, respectively.

12.3.2 Chemical Pretreatments

12.3.2.1 Alkali

This method involves the use of bases such as ammonium, sodium, calcium and potassium hydroxides for the pretreatment of lignocellulosic biomass. Alkaline pretreatment leads to breakdown of ester and glycosidic side chains which results in the disruption of lignin and hemicellulose, cellulose swelling and partial decrystallization of cellulose. Sodium hydroxide (NaOH) has been extensively used. It has been shown to disrupt the lignin present in the biomass, which allows increased accessibility of enzymes to cellulose and hemicellulose. Temperature, time, liquid-solid ratio and NaOH concentration are the parameters that affect the pretreatment process. Various lignocellulosic feedstocks are amenable to this pretreatment method. Dilute NaOH pretreatment has no effect on softwoods as they have higher lignin content (>30%) (Kumar and Wyman 2009).

The alteration in the composition of rice husk was also observed following NaOH pretreatment. The alkaline pretreatment increased the cellulose content to 49.02% from 37.55% in untreated rice husk. The hemicellulose and lignin contents were also found to decrease to 3.81% and 6.61%, respectively as compared to these of untreated rice husk (Nikzad et al. 2014). Alkali pretreatment along with microwave treatment led to increase in the removal of lignin and hemicellulose, besides enhancement in hydrolysis rate and glucose content (Zhu et al. 2005).

12.3.2.2 Acid

Sulfuric acid (H_2SO_4) and hydrochloric acid are the most commonly used mineral acids for the chemical treatment of lignocellulosic biomass. Acid pretreatment is carried out either under high acid concentration and low temperature or low acid concentration and high temperature. The addition of H_2SO_4 removes hemicellulose, which enhances digestibility of cellulose in the residual solids. The parameters such as acid concentration, particle size, liquid to solid ratio, retention time and temperature affect acid pretreatment process. This method has few major limitations including toxicity of the acids, corrosiveness of equipments, acid recovery, pH neutralization of the biomass and production of fermentation inhibitors HMF and 2-furfuralaldehyde. Dilute acid has been found to be more effective as it generated lower amount of fermentation inhibitors. There are also a few reports on the combination of both acid and alkali, which led to higher sugar yields as it caused removal of both hemicelluloses and lignin by acid and alkali pretreatments, respectively.

Rice straw pretreated with 1% (w/w) H_2SO_4 for 1–5 min at 160 °C led to maximal sugar yield of 83% (Hsu et al. 2010). After hydrogen peroxide and acetic acid pretreatment, 97.2% of the lignin was removed from lignocellulosic biomass. Maximum theoretical yield of 85% and 412 mL ethanol kg^{-1} of biomass was attained by fermentation of the hydrolysates for 24 h (Wi et al. 2015). Vasconcelos et al. (2013) reported that 0.20% phosphoric acid concentration at 186 °C for 8 and 24 min resulted in 96% and 98% solubilization of hemicelluloses from sugarcane bagasse, respectively. Enzymatic hydrolysis resulted in 367 g glucose/kg of the

pretreated bagasse, which accounts for the conversion of 56.38% cellulose to glucose. The combined alkali and acid treated lignocellulosic biomass yielded more glucose (Lee et al. 2015; Zhao et al. 2009; Kim et al. 2012).

12.3.2.3 Organosolv

Organosolv process involves the use of an organic solvent or mixtures of organic solvents with water for removal of lignin and hemicellulose. Organic solvent cleaves the internal bonds between lignin and hemicelluloses. Commonly used solvents to pretreat different lignocellulosic materials include ethanol, methanol, acetone, ethylene glycol, and N-methylmorpholine-N-oxide (NMMO or NMO). Temperature used for the process is dependent on the type of biomass and the catalyst (organic/inorganic acids and bases) used; usually high temperature (~200 °C) is used (Ghose et al. 1983). Solvents must be removed as it can be an inhibitor for enzymatic hydrolysis and fermentation process. Solvent recovery, high cost (in some cases), inflammability and volatility can be considered as its drawbacks. Temperature, retention time and solvent concentration are some of the parameters that affect the pretreatment.

Two-fold increase in ethanol production was attained in sorghum bagasse pretreated with 1-butanol or 1-pentanol in comparison with the untreated biomass (Teramura et al. 2016). Organosolv pretreatment along with other pretreatment methods has also been found to be efficient (Mesa et al. 2011; Brosse et al. 2010). Dilute acid pretreatment of sugarcane bagasse followed by the organosolv (ethanol) pretreatment with NaOH for 60 min and 195 °C using 30% (v/v) ethanol; the yield of 29.1 g glucose/100 g sugarcane bagasse was attained (Mesa et al. 2011). Salapa et al. (2017) investigated the organosolv pretreatment of wheat straw using five solvents (ethanol, methanol, butanol, acetone and diethylene glycol). A maximum cellulose conversion of 89% was found with ethanol pretreatment at 180 °C for 40 min. The ethanol concentration of 32.59 g/L was achieved, which accounted for 67% of theoretical yield.

12.3.3 Ozonolysis

This pretreatment involves the use of ozone gas as an oxidant which attacks lignin and increase cellulose biodegradability. Ozonolysis is performed at ambient temperature and pressure, unlike other chemical pretreatment methods. This method is environment friendly as it does not produce any toxic inhibitors and it does not affect enzymatic hydrolysis and fermentation process. Moisture content is the important factor that affects the ozone pretreatment. Higher moisture content leads to low lignin oxidization. This pretreatment method is less suitable and costly at industrial scale due to high amount of ozone requirement. The major limitation with this method is its cost, as the high amount of ozone is required which makes it an expensive and a less suitable option for pretreatment at industrial scale. Several agricultural residues such as wheat straw (Garcia-Cubero et al. 2009), bagasse (Travaini et al. 2013), peanut and poplar sawdust (Vidal and Molinier 1988) and

olive mill waste (Benitez et al. 1997) have been pretreated by this method. Sugar yields of 88.6% and 57% were attained after pretreatment as compared to 29% and 16% of the untreated wheat and rye straw, respectively (Garcia-Cubero et al. 2009). In sugarcane bagasse, the yield of glucose and xylose increased from 6.64% to 46.06% and 2.05% to 52.44%, respectively (Travaini et al. 2013). In case of poplar sawdust, lignin content was reduced to 8% with sugar yield increased to 57% (Vidal and Molinier 1988).

12.3.4 Ionic Liquid

Ionic liquids (IL) are the organic salts, which are liquid at low temperatures. Degradation of the complex network of cellulose, hemicellulose and lignin takes place due to hydrogen bonding between the non-hydrated ions of ILs and the sugar hydroxyl protons. Disadvantages associated with IL are that they become more viscous during the pretreatment, expensive and toxic to hydrolytic enzymes. The most commonly used IL for the pretreatment of lignocellulosics are1-allyl-3-methylimidazolium-chloride ([AMIM]Cl,1-ethyl-3-methylimidazoliumdiethyl-phosphate ([EMIM]DEP), 1-butyl-3methylimidazoliumchloride ([BMIM]Cl, 1-butyl-3-methy-limidazolium hexafluorophosphate, 1-butyl-3-methylimidazoliumacetate, 1-benzyl-3-methylimidazoliumchloride,1-butyl-1-methylpyrrolidinium-chloride, 1-butyl-3-methylimidazoliummethylsulfate, N,N-dimethylethanolammonium groups, 1-ethyl-3-methylimidazolium groups and 1,3-dimethylimidazolium groups. Major factors affecting interaction between IL and lignocellulosic materials are cations, anions, temperature and time used in the pretreatment process.

Glucose (90%) and hemicellulose (25%) present in the original biomass were released by enzymatic hydrolysis of pine and willow biomass pretreated with combined ionic liquid pretreatment (mixtures of 1-butyl-3-methylimidazolium methylsulfate and 1-butyl-3-methylimidazolium hydrogen sulfate). It was found that ionic liquors containing methylsulphate, hydrogensulphate and methanesulfonate anions were most effective for cellulose digestibility (Brandt et al. 2011). The saccharification of sugarcane and enzyme digestibility of oil palm empty fruit bunches of biomass pretreated with 1-ethyl-3-methylimidazolium [C2MIM]Ac increased from 53% to 83% and from 20.4% (untreated control) to 96.6% respectively as compared to pretreatment achieved by [C_2MIM]Cl and [C_4MIM]Cl (Karatzos et al. 2012; Katinonkul et al. 2012).

12.3.5 Biological Pretreatment

In comparison with conventional chemical and physical pretreatment methods, biological pretreatment is considered as an efficient, environmentally safe and low-energy process. Other advantages of biological pretreatment include mild reaction conditions, higher product yields, fewer side reactions, less energy demand and less reactor resistance to pressure and corrosion. Various cellulolytic and hemicellulolytic

Table 12.4 Effect of various pretreatment methods on lignocellulosic biomass

	Milling	Steam explosion	Acid	Alkaline	Oxidative	Lime
Increases accessible surface area	H	H	H	H	H	H
Cellulose decrystallization	H	–	–	–	ND	ND
Hemicellulose solubilization	–	H	H	L	–	M
Lignin removal	–	M	M	M	M	H
Generation of toxic compound	–	H	H	L	L	M
Lignin structure alteration	–	H	H	H	H	H

Adapted from Mosier et al. (2005)
H High, *M* Medium, *L* Low, *ND* Not Determined

microbes can be used for effective biomass pretreatment (Vats et al. 2013). Biological pretreatments are carried out by using ligninolytic enzymes (laccases, manganese peroxidases and lignin peroxidases) or microorganisms such as brown, white, and soft-rot fungi which mainly degrade lignin and hemicellulose and little amount of cellulose (Sánchez 2009). The white-rot fungal species commonly employed in biological pretreatment include *Phanerochaete chrysosporium, Ceriporia lacerata, Cyathus stercoreus, Ceriporiopsis subvermispora, Pycnoporus cinnarbarinus* and *Pleurotus ostreaus.*

Glucose yields by treatment of sugarcane bagasse with *C. submervispora* were comparable with those from physico-chemical pretreatments. The pretreatment with *C. subvermispora* preserved most of the glucan fraction with improved cellulose digestibility (Machado and Ferraz 2017). Saha et al. (2016) reported *Cyathus stercoreus* NRRL-6573 to yield higher sugar (394 ± 13 mg/g) from the pretreated corn stover followed by *Pycnoporus sanguineus* FP-10356-Sp (393 ± 17 mg/g) and *Phlebia brevispora* NRRL-13108 (383 ± 13 mg/g).

There are reports on the effects of different pretreatments on biomass composition and sugar yields (Eggeman and Elander 2005). The selection of an efficient pretreatment method varies with the biomass be treated because of their varied composition and should be based on the attributes such as less loss of sugar, cost effective, high lignin removal capability, increase in accessible surface area, no/less generation of toxic compounds. Table 12.4 summarizes the effect of different pretreatment technologies on the structure of lignocellulose.

12.4 Cellulases

12.4.1 Cellulases and Their Classification

Cellulases are glycoside hydrolases which catalyze hydrolysis of the glycosidic linkages in glycosides, leading to the formation of a sugar hemiacetal or hemiketal and the corresponding free aglycon. Glycoside hydrolases (GH) have been classified

into 115 families based on amino acid sequence similarities and crystal structures. The Carbohydrate-Active Enzymes database (CAZy; http://www.cazy.org) provides continuously updated information of the glycoside hydrolase (EC 3.2.1.) families (Cantarel et al. 2009). Cellulases are found in GH 13 families (1, 3, 5, 6, 7, 8, 9, 12, 26, 44, 45, 51, and 48); cellulase like activities have also been proposed for GH families 61 and 74 (Schulein 2000). CBHs are the most-studied exoglucanase, which accounts for about 70% of the secreted cellulases. Cellobiohydrolases (CBHs) and endoglucanases (EGLs) are produced by various bacteria and fungi, with catalytic modules belonging to GH families 5, 6, 7, 9, 48, 74 and 5, 9, 12, 44, 45, 48, 51, 74, respectively. The β-glucosidases (BGLs) belongs to catalytic modules of families 1, 3, and 9. The protein in each GH family is related by sequence, and by corollary, folding. Based on the data in CAZy database, the three dimensional structures of most of the cellulases are known. Cellulases display a variety of topologies ranging from all β-sheet proteins to β/α-barrels to all α-helical proteins.

Soils harbor microbes capable of producing cell wall degrading enzymes, which hydrolyze the complex components of cell wall into simple sugar molecules. Most organisms isolated from soil/composting waste materials are capable of producing a wide variety of cell wall degrading enzymes. Fungal cellulases are modular enzymes with functionally distinct modules or domains. Most of the cellulases are composed of carbohydrate binding modules (CBM) and catalytic domain connected by serine and threonine rich flexible linker protein. CBMs are 4–20 kDa in size and are rich in aromatic and polar amino acid residues which bind to the substrate during catalysis. CBMs play a major role in increasing the binding affinity between cellulases and cellulose substrate.

Cellulases consist of three major components: (1) endo-β, 1–4-glucanases (endo-β, 1–4-d-glucan 4-glucanohydrolase, EC 3.2.1.4), (2) exo-β, 1–4-glucanase or cellobiohydrolase (exo-β, 1–4-d-glucan 4-cellobiohydrolase, EC 3.2.1.91), and (3) β-glucosidase (EC 3.2.1.21). These three components act synergistically to hydrolyze cellulose to glucose, therefore cellulases are known as multienzyme complexes. Some cellulase systems also comprise exo-β, 1–4-D-glucan 4-glucohydrolase (EC 3.2.1.74) and exo-β, 1-4-cellobiosidase (EC 3.2.1.176)]. As shown in Fig. 12.1, endoglucanases hydrolyze the amorphous (internal) regions by cleaving β-1,4 linkages, while cellobiohydrolases are exoglucanases which cleave disaccharides from non-reducing end by releasing cellobiose, while cellobiose and other cellodextrins are hydrolyzed by β-glucosidases into individual monomeric units of glucose.

The enzymes from GH family 61 display lytic polysaccharide monooxygenase activity (LPMO), which enhance cellulolytic activity when mixed with common cellulases, thus known as ‘cellulase enhancing factors’ (Horn et al. 2012). LPMOs are copper-dependent enzymes, which cleave cellulose by an oxidative mechanism. They are classified in AA9 family of CAZy database (Langston et al. 2011). LPMOs act on amorphous cellulose surfaces and induce weakening of the hydrogen bonds and van der Waals network linking adjacent cellulose chains that result in chain breakage and associated chemical modification. Crystalline cellulose structure is, therefore, disrupted and becomes less ordered (Villares et al. 2017). Spiking of

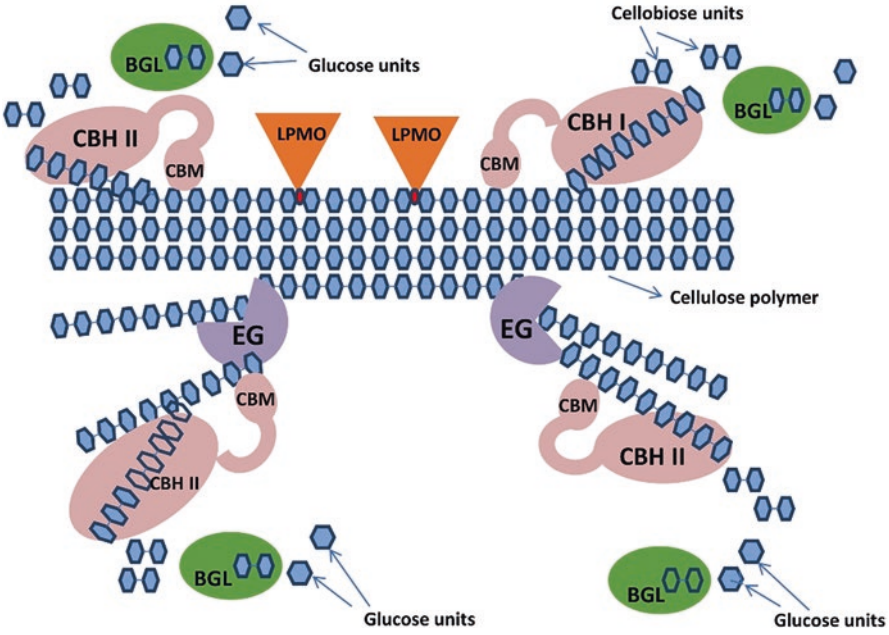

Fig. 12.1 Cellulose degradation by cellulolytic and other accessory enzymes (Singh et al. 2017b). (*EG* endo-β-1,4-endoglucanase, *CBH* cellobiohydrolase, *BGL* β–glucosidase, *CBM* carbohydrate binding module, *LPMO* lytic polysaccharide monooxygenase)

traditional cellulase cocktails with LPMOs lead to increase in saccharification yields (about 60% more glucose) (Muller et al. 2015).

Cellulases find applications in food, animal feed, textiles as well as in paper and pulp industries. Study of cell wall degrading enzymes of microbial origin is an area of intensive research. A large number of microorganisms (bacteria, actinobacteria and fungi) are known for their ability to hydrolyze lignocellulosics. Most commonly used hydrolytic enzymes producing microbes are *Pseudomonas, Clostridium, Bacillus, Aspergillus, Trichoderma* and *Penicillium*. Thermophilic fungi like *Myceliophthora thermophila*, *Thermoascus aurantiacus*, *Humicola insolens* and *Talaromyces emersonii* are good producers of thermostable cellulases (Singh et al. 2016, 2017a).

12.4.2 Cellulase Production by Wild Microbial Strains

In the past few years, various bacteria and fungi have been studied widely for the production of cellulolytic enzymes. Mainly, fungi have received considerable attention because of their ability to secrete copious amounts of cellulolytic enzymes with less complexity as compared to bacterial cellulases. Table 12.5 lists some of the cellulase producing microorganisms.

Table 12.5 Cellulase producing microorganisms (Kubicek 1993)

Bacteria	Fungi	Actinobacteria
Clostridium thermocellum	*Talaromyces emersonii*	*Streptomyces* spp.
Ruminococcusalbus	*Thielavia terrestris*	*Thermoactinomyces* spp.
Streptomyces reticuli	*Trichoderma koningii*	*Thermonospora curvata*
Bacillus pumilis	*Trichoderma reesei*	
Acidothermus cellulolyticus	*Trichoderma viride*	
Pseudomonas fluorescens	*Sporotrichum cellulophilum*	
Eubacterium cellulosolvens	*Sporotrichum thermophile*	
Spirochaeta thermophila	*Fusarium solani*	
Fibrobacter succinogenes	*Penicillium funiculosum*	

It has been reported that cellulolytic activity of fungi is affected by culture conditions and medium composition. Therefore, in order to achieve enhanced productivity, various synthetic or natural carbon sources have been employed. Badhan et al. (2007) studied the production of cellulase by *Myceliophthora* sp. IMI 387099 using different carbon sources (rice straw [RS], wheat straw [WS], sugarcane bagasse [SB], corn cobs [CC] and wheat bran [WB]). RS supported a high production of endoglucanase (32 U/gds), β-glucosidase (7.48 U/gds) and FPase (2.44 U/gds). A basal medium comprising $(NH_4)_2SO_4$, CH_3COONH_3 and KH_2PO_4 supported a high enzyme production. The high level production of cellulolytic enzymes from *Inonotus obliquus* has been studied for the first time by Xu et al. (2017). Various lignocellulosic substrates were screened for the production of cellulases in solid-state fermentation (SSF); WB supported greater production of CMCase (17.66 IU/g) and β-glucosidase (1.71 IU/g). The enzyme exhibited thermal stability upto 70 °C and tolerance towards acidic pH. Enzymatic saccharification released reducing sugars from RS (125.36 mg/g of substrate) and WS (130.24 mg/g of substrate) after 48 h of hydrolysis. Different SSF parameters were optimized for the production of cellulases and xylanase by *Aspergillus terreus* M11 (Gao et al. 2008). Mixed substrates (RS and WB) were also used for producing cellulase by *Aspergillus fumigatus* ABK9 (Das et al. 2013). Fermentation time, pH of the medium, substrate concentration and substrate ratio significantly influenced enzyme production. Under optimum conditions, endoglucanase, β-glucosidase and FPase activities of 826.2, 255.16, and 102.5 U/g were attained, respectively (Das et al. 2013). Endoglucanase, β-glucosidase and FPase activities of 581, 128 and 243 U/g of carbon source were achieved at temperature 45 °C, pH 3 and moisture 80% with corn stover and 0.8% yeast extract as carbon and nitrogen sources. Maximum activities of endoglucanase and β-glucosidase were seen at pH 2.0 and pH 3.0, respectively; both the enzymes exhibited stability in the range of pH 2.0–5.0 at 70 °C (Gao et al. 2008). The optimization of the nitrogen source (7%), initial moisture level (80%), growth temperature (33 °C) and initial pH (7) of the culture medium led to enhanced cellulolytic and xylanolytic enzyme production levels by *Fusarium oxysporum* in SSF on corn stover (Panagiotou et al. 2003). Endoglucanase, cellobiohydrolase and β-glucosidase production of 211, 3.9 and 0.088 U/g of substrate were recorded.

Trichoderma reesei is one of the most exploited fungal strains for cellulase production (Ross et al. 1983; Domingues et al. 2000; Liming and Xueliang 2004; Satyamurthy et al. 2016).

12.4.3 Cellulase Production by Recombinant Strains

Various genetic approaches have been investigated over the last few decades to enhance cellulolytic enzyme production and expression levels. Various genetically modified microbial strains like *T. reesei*, *A. niger*, *P. pastoris*, *S. cerevisae*, *E. coli,* and *B. subtilis* have been employed for expressing heterologous genes of commercial significance. The conventional method used for strain improvement is by random mutagenesis. Tambor et al. (2012) screened 55 fungal endoglucanases for secretion at high levels in *Aspergillus niger.* Three endoglucanases from *Aureobasidium pullulans* (ApCel5A), *Gloeophyllum trabeum* (GtCel12A) and *Sporotrichum thermophile* (StCel5A) have been identified. A high level of expression (≥0.3 g/L) was attained in *A. niger.* ApCel5A and GtCel12A were acidstable, whereas StCel5A displayed thermostability. All three enzymes exhibited significant synergism with a major exoglucanase from *Trichoderma reesei* TrCel7A. Phadtare et al. (2017) reported constitutive expression of codon optimized *M. thermophila* endoglucanse gene (*Mt-egl*) in *P. pastoris* extracellularly for the first time. The recombinant endoglucanase (rMt-egl) is a monomeric protein of ~47 kDa with $T_{1/2}$ of 60 and 15 min at 90 and 100 °C, respectively. Homology modeling confirmed that glutamate 234 and 344 are catalytically important amino acid residues. By the action of rMt-egl on wheat bran and corn cobs, reducing sugars liberated were 421 mg/g and 382 mg/g, respectively.

There are several reports on recombinant bifunctional cellulase-xylanase genes too (Prabhu et al. 2017; Rattu et al. 2016; Xue et al. 2017; Rizk et al. 2015). A bifunctional endoglucanase gene (BhCellXyl) of polyextremophilic bacterium *Bacillus halodurans* TSLV1 was expressed in *E. coli* (Rattu et al. 2016) and *P. pastoris* under constitutive *GAP* as well as inducible *AOX* promoters (Prabhu et al. 2017). The *E. coli* recombinant (rBhcell-xyl) showed CMCase activity (2272 U/L) and xylanase activity (910 U/L) (Rattu et al. 2016). A higher titer of recombinant BhCell-Xyl was attained (4.8 U/ml) when cloned under *AOX* promoter as compared to that under *GAP* promoter (2.1 U/ml). The glycosylated BhCell-Xyl exhibited higher thermostability than the native enzyme. The *in silico* analysis revealed that BhCell-Xyl has one active site for both endocellulase and endoxylanase activities (Prabhu et al. 2017). The cellulase from *Bacillus amyloliquefaciens* was expressed in *A. niger* which showed enhanced exoglucanase activity from 0.21 U/ml to 0.89 U/ml and endoglucanase activity from 4.51 U/ml to 15.12 U/ml as compared to that with the native strain. The glucose yield by hydrolysis of wheat straw was also 1.37-fold higher than that with cellulase from the native strain (Xue et al. 2017). Some reports also suggest that removal or addition of glycan sites may enhance the cellulase activity. Enhanced enzyme activity (35%) was observed by removal of three *N*-glycan sites from rCel7A of *Penicillium funiculosum.* While enhanced

enzyme activity (85%) was observed by addition of *N*-glycan site on asparagine-194 via mutation of alanine-196 to serine in *A. niger* (Adney et al. 2009). A gene encoding β-glucosidase (*cel3a*) from a thermophilic fungus *Talalaromyces emersonii* was expressed in *Trichoderma reesei*. The enzyme Cel3a was found to be a homodimer of 130 kDa. The enzyme was acid stable and thermostable with temperature optimum of 71 °C. Because of glycosyltransferase activity, mainly cellobiose from glucose and cellotetraose from cellobiose were produced (Murray et al. 2004).

The heterologous expression of a gene is often affected by the codon bias, which causes inadequate translation of target gene, which results in low or absence of heterologous expression. In order to overcome this, codon optimization of target genes (cellulases) is also one of the strategies which have received attention in the past few years. Endoglucanase 1 (*egl1*) and *cbh2* gene from *T. reesei* were codon optimized and expressed in active form in *P. pastoris* under *AOX* promoter. 1.24-fold enhancement in EG1 production (Akcapinar et al. 2011) and two-fold increase in cbh2 enzyme production (Fang and Xia 2015) were attained with synthetic codon optimized gene as compared to the native strain.

Although heterologous fungal proteins can be produced in grams/Litre quantities, the yields of heterologous gene products are low (Gibbs et al. 2004). The main limitation that leads to low yields lies in the processing/secretion of the proteins (Nyyssőnen et al. 1993). In order to improve the processing and yields of biotechnologically useful heterologous proteins in fungi, it is necessary to understand gene regulation (Tèo et al. 2000), protein glycosyation (Wang et al. 1996), occurrence of intra- and extra-cellular proteases and application of proteomics of fungi (Lim et al. 2001).

12.5 Immobilization of Cellulases

The major limitation in lignocellulosic ethanol production is the high cost of enzymes and fermenting microbes. The reuse of enzymes and microorganisms can reduce the cost involved. Immobilization of enzymes and cells on inert supports can be one of the strategies to deal with such issues. This strategy permits not only recycling of enzymes but also increases stability and thermal characteristics of the enzymes. Usually enzymes can be immobilized on solid particles and cross-linking techniques. Various techniques of immobilization include adsorption onto a solid carrier, entrapment in a polymer matrix, affinity interactions, covalent linking to a solid support and crosslinking of enzyme aggregates (CLEAs) or their combination. The major advantages of immobilizing enzymes are increased ethanol yield, reduced risk of microbial contamination due to high cell densities, recycling in repeated batch fermentations, easier product recovery, reduced end-product inhibition, less energy demands and process expenses, regeneration and reuse for extended periods (Zhu 2007). Yao et al. (2011) studied yeast cell immobilization by entrapment in the porous network of bacterial cellulose membrane (BCM). The observed specific rate of ethanol production by immobilized cells in BCM was 2.1 g/g with high ethanol

yield (9.7% v/v). The ethanol yield observed was 21.25% higher as compared to the free cells. In another study, cellulase was immobilized onto silica gel surfaces pretreated with (3-aminopropyl) triethoxy-silane (3-APTES) and glutaraldehyde (GA) as cross-linker. Initial activity of immobilized cellulase was 48% after 4 days, and 22% after 14 days (Zhang et al. 2016). Cellulase produced by *Aspergillus fumigatus* was immobilized on MnO_2 nanoparticles. The enzyme immobilization led to enhanced enzyme activity, thermostability and bioethanol production (21.96 g/L) as compared to that with free enzymes (18 g/L) (Cherian et al. 2015). Immobilization of cellulase on the activated magnetic nanoparticles encapsulated in polymeric nanospheres via glutaraldehyde activation (Lima et al. 2017) and on poly (acrylic acid) [PAA] nanogel (Ahmed et al. 2017) led to improvement in thermostability, acid stability, and reusability. At 80 °C, the PAA immobilized cellulase retained ~75% hydrolytic activity as compared to ~55% with the free cellulase. The reuse of enzymes through immobilization is a well-known method towards recycling. There are certain drawbacks of using carrier bound enzymes mainly, the high cost of the matrices (carrier), mass transfer and diffusion problems, clogging of filters during separation and downstream processing and the presence of large amount of non-catalytic mass (usually>90% that of the enzyme) (Terrasan et al. 2016). Immobilization of cross-linked enzyme aggregates (CLEA) has been attempted recently (Bhattacharya and Pletschke 2014; Periyasamy et al. 2016; Perzon et al. 2017), which allows carrier-free immobilization. Khorshidi et al. (2016) studied immobilization on the amine-functionalized Fe_3O_4 on silica core-shell magnetic nanoparticles (MNPs). Cellulase CLEA-MNP retained about 45% and 65% of its maximum activity at pH > 4.8 and 65 °C, respectively; while free cellulase lost its activity. Immobilization leads to increase in thermostability of cellulases (Zang et al. 2014). As the degradation of cellulase requires the complex mixtures and synergistic action of various cellulases, xylanses and other enzymes, this technique may also offer the combination of enzymes. Periyasamy et al. (2016) attempted this approach in using combi-CLEAs with xylanase, cellulase and β-1,3-glucanase. Combi CLEAs led to maximum hydrolysis of about 83.5% in 48 h, whereas free enzymes caused only 73% hydrolysis.

12.6 Microbial Strains for Fermenting Cellulosic Hydrolysates to Ethanol

Ethanol fermentation is a biological process in which lignocellulose biomass is hydrolyzed to simple sugars using enzymes; the sugar produced is then fermented to ethanol by ethanologenic microbes. The important characteristics of an ethanologenic microbe are high ethanol yield, capability to utilize multiple sugars, high ethanol productivity, tolerance to high ethanol concentration and inhibitors. Various microorganisms including bacteria, fungi and yeasts play a potential role in fermentation of soluble sugars in the absence of oxygen, which results in the production of ethanol. Due to their varied efficiency and feasibility in fermentation, only a limited number of microbes are used for large scale ethanol production. Table 12.6 lists microbes used in fermenting glucose to ethanol (Binod et al. 2013).

Table 12.6 Microbes that ferment hexose sugar (glucose)

Bacteria	Fungi	Yeast
Zymomonas mobilis	*Mucor indicus*	*Saccharomyces cerevisiae*
Esherichia coli	*Aspergillus terreus*	
Clostridium thermocellum	*Fusarium oxysporum*	
Clostridium phytofermentans	*Neurospora crassa*	
Klebsiella oxytoca		
Clostridium sporogenes		
Thermanerobacter ethanolicus		
Pachysolen tannophilus		

The most commonly used microbe is the yeast (*Saccharomyces cerevisiae*). It is efficient for the fermentation of six-carbon sugars to ethanol concentration as high as 18% of the fermentation broth. It metabolizes glucose by the Embden-Meyerhof (EM) pathway. Among bacterial species, *Zymomonas mobilis* is widely used for ethanol fermentation due to its high ethanol yield (up to 97% theoretical yield) and high ethanol tolerance (14% v/v). It is the only bacterium that uses Entner-Doudoroff (ED) pathway instead of EM or glycolytic pathway to metabolize glucose anaerobically. ED pathway yields only half as much ATP per mole of glucose, which results in less biomass and more yields as compared to yeasts. The emphasis has been to construct an efficient organism through metabolic engineering, due to the absence of natural microorganisms for efficient fermentation of lignocellulosic biomass. This involves optimizing existing biochemical pathways or introducing pathway components, which imparts additional features such as ethanol tolerance, high ethanol productivity and yield and co-fermentation of both glucose and xylose. Much work has been done on metabolic engineering of microbes. The expression of pyruvate decarboxylase and alcohol dehydrogenase II genes from *Z. mobilis* into *E. coli* under the control of a lac promoter resulted in a high ethanol yield due to co-fermentation of hexose and pentose sugars (Ohta et al. 1991). Recently, it was shown that overexpression of the autologous GroESL chaperone system resulted in a 12-fold increase in cell growth in presence of 4% (v/v) ethanol as compared to the native strain (Zingaro and Papoutsakis 2013). *S. cerevisiae* (strain DA24–16) was engineered by expressing genes encoding cellodextrin transporter and a β-glucosidase (*cdt-1 and gh1-1*) to construct a strain capable of consuming cellobiose and xylose simultaneously. The recombinant strain was able to consume 80 g/L of cellobiose/xylose mixture within the same period as that required to consume 40 g/L of cellobiose or 40 g/L xylose separately; ethanol productivity also dramatically increased from 0.27 to 0.65 g/L/h during co-fermentation (Ha et al. 2011). Efforts have also been made to minimize furfural tolerance (Wang et al. 2013; Tan et al. 2015) in fermentation process, as furfural from lignocellulosic hydrolysates is the key inhibitor for ethanol fermentation. Tan et al. (2015) reported a strategy of improving furfural tolerance in *Z. mobilis* by engineering its global transcription sigma factor (σ70, RpoD) protein. Three furfural tolerance RpoD mutants (ZM4-MF1, ZM4- MF2, and ZM4-MF3) were

identified; ZM4-MF2 was found to be best furfural-tolerant strain, which also consumed glucose faster with high ethanol yield (Tan et al. 2015).

Intensive efforts are called for studying metabolic pathways in detail so as to identify various genes, which could be overexpressed or knocked out for understanding the interactions among various pathways to enhance ethanol production. This is expected to develop recombinants with the desirable characteristics.

12.7 Fermentation Strategies

Requirement of different technological steps involved in the conversion of lignocellulosics to bioethanol, such as pretreatment, detoxification, hydrolysis and fermentation has made the conversion process more complex. The integration of these processing steps has been attempted for reducing energy consumption, capital investments and process time. Typically, separate hydrolysis and fermentation (SHF) is followed. The advantage of using this method is the feasibility to operate hydrolysis and fermentation under their optimum conditions, but the major drawback is feedback inhibition of enzymes due to accumulation of sugars in hydrolysates. Simultaneous saccharification and fermentation (SSF) is promising process integration in ethanol production. SSF is referred to as cellulose hydrolysis and fermentation of sugars simultaneously in a single reactor that significantly reduces the product inhibition of hydrolysis. Saha et al. (2015) reported production of ethanol by recombinant bacterium from lignocellulosic biomass for the first time. Theoretical ethanol yield of 57% (0.29 g/g of ethanol) was recorded using recombinant *E. coli*. Using cellulase produced from *Penicillium oxalicum* EU2106, the glucan conversion of sugarcane bagasse (SB) reached 93.36% after 96 h hydrolysis of sugarcane bagasse pulp. Following SSF, the ethanol yield of 18.79 g/L was produced by thermotolerant *Saccharomyces cerevisiae* ZM1–5 (Huang et al. 2015). A thermotolerant *S. cerevisiae* strain exhibited 80.65% and 73.5% ethanol yield with high solid loading (20%) and 20 FPU/g by SSF and SHF, respectively (Mishra et al. 2016). SSF combined with co-fermentation of hexose and pentose sugars is called simultaneous saccharification and co-fermentation (SSCF). Using genetically modified pentose fermenting *S. cerevisiae* strain (KE6–12), co-fermentation of glucose and xylose was demonstrated. Highest ethanol concentration attained was 37.5 g/L that corresponds to an overall ethanol yield of 0.32 g/g in SSCF (Bondesson and Galbe 2016). Using SHF, the ethanol yield of 0.38 g ethanol/g sugar was recorded from waste paper. The mixture of cellulolytic enzymes (produced locally by *Trichoderma reesei* Rut-C30 and *Aspergillus niger* F38) were used for hydrolysis. Under optimal hydrolysis conditions, the saccharification of newspaper and office paper were 67% and 92%, respectively (Guerfali et al. 2015).

Cellulase production by solid state fermentation is also gaining attention. It is a fermentation process with almost no free water, but substrate moist enough to support growth of microorganisms. The process mimics the natural habitat of most filamentous fungi as compared to submerged fermentation (SmF). Solid state fermentation is, therefore, preferred over submerged fermentation. The advantages

of solid state fermentation include lower processing cost, less energy requirement, enhanced productivity, improved product stability and minimization of catabolite repression. In an investigation, Trivedi et al. (2015) produced cellulases from the marine fungus *Cladosporium sphaerospermum* in solid state fermentation, which was used in saccharification of seaweed biomass *(Ulva fasciata).* The ethanol yield of 0.47 g/g reducing sugar, corresponding to 93.81% conversion efficiency was recorded. Deswal et al. (2011) reported an initial pH of 5.5 and moisture ratio of 1:3.5 (solid:liquid) for enhanced cellulase production by the brown rot fungus *Fomitopsis* sp. (RCK2010). Out of various carbon sources tested, maximum enzyme production of CMCase (71.526 IU/g), FPase (3.268 IU/g), and β-glucosidase (50.696 IU/g) was attained in wheat bran. Reducing sugars released after saccharification of pretreated rice straw and wheat straw by crude enzyme extract were 157.16 mg/g and 214.044 mg/g, respectively.

Ethanol concentration achieved so far is low for industrial scale economical production. Future research should focus on increasing the rate of enzymatic hydrolysis. Improvements are, therefore, required in the processes to develop a commercially viable technology for the production of ethanol. In the past few years, extensive efforts have been made to ferment both glucose and xylose (Goncalves et al. 2014; Krahulec et al. 2010; Agbogbo et al. 2006). Recombinant DNA technology allows genetic changes in bacteria and yeasts for enabling the recombinants to ferment both xylose and glucose simultaneously to ethanol.

12.8 Metabolic Engineering for Efficient Ethanol Production

In order to improve ethanol yield and production, metabolic engineering of ethanologenic microbes was performed using two main strategies: introduction of heterologous gene(s), and disruption of genes involved in byproduct formation that competes with ethanol synthesis. Ethanol can be produced from pyruvate via two pathways (i) oxidative decarboxylation of pyruvate and subsequent acetyl-CoA reduction to acetaldehyde and then to ethanol; (ii) the pyruvate decarboxylase (Pdc) catalyzed conversion of pyruvate to acetaldehyde which is reduced by alcohol dehydrogenase (Adh). The expression of *Zymomonas mobilis Pdc* and *Adh* genes led to improved ethanol production. The strategy was applied to *Clostridium cellulolyticum*; the ethanol concentration increased by 53% as compared to the wild strain (Guedon et al. 2002). Deletion of β-hydroxybutyryl-CoA dehydrogenase (*hbd*) could also be applied in butyrate producing cellulolytic strains such as *C. cellulovorans* and *C. thermopapyrolyticum.* Ethanol titer obtained by *hbd*-deficient *C. butyricum* was 18-fold higher than in the wild type (WT) strain (Cai et al. 2011). Deletion of acetate kinase (*ack*), phosphate acetyltransferase (*pta*), and L-lactate dehydrogenase (*ldh*) genes in hemicellulolytic *T. saccharolyticum* strain led to maximum ethanol productivity of 2.2 g/L/h and titer up to 65 g/L, respectively (Shaw et al. 2008). Strain improvement in *S. cerevisiae* through mutations in *SPT15* gene (a transcription regulator) resulted in ethanol tolerance (Alper et al. 2006). In *Thermoanaerobacterium saccharolyticum*, production of lactic acid has

been completely eliminated by knocking out *ldh* gene that led to increase in the yield of ethanol (Desai et al. 2004). The metabolic engineering also aids in developing an efficient consolidated bioprocessing (CBP) platform for biofuel production from cellulosic biomass. A few recent investigations carried out on metabolic engineering of various microbes in order to achieve high ethanol yields are summarized in Table 12.7.

Table 12.7 Recent investigations on metabolic engineering of microbes for improving ethanol production

Organism	Metabolic engineering strategy	Description	References
Clostridium thermocellum	Deletion of type I glutamine synthase (glnA) gene	Ethanol yield increased to 53%	Rydzak et al. (2017)
Fusarium oxysporum	Overexpression of transaldolase (tal) and phosphoglumutase (pgm)	Ethanol yield increased from 0.146 to 20.9 g/L as compared to WT	Anasontzis et al. (2016)
Saccharomyces cerevisiae	Deletion of aldolasereductase (GRE)	Ethanol yield increased (0.47 g/g)	Romani et al. (2015)
		Decreased xylitol production	
Clostridium cellulovorans	Expression of aldehyde alcohol dehydrogenase (adhE2) gene	Ethanol production directly from cellulose (1.60 g/L)	Yang et al. (2015)
Clostridium thermocellum	Knockout hydrogenase gene	Ethanol yield increased 90% compared to WT	Biswas et al. (2015)
		Acetic acid production decreased to 74%	
Cornyebacterium glutamicum	Overexpression of glycolytic genes (pgi, pfkA, gapA, pyk)	Ethanol productivity increased 1.7-fold	Jojima et al. (2014)
		Glucose consumption increased 1.9-fold	
Thermoanaerobacterium saccharolyticum	Expression of urease gene	Increased ethanol yield (54 g/L)	Shaw et al. (2012)
Clostridium thermocellum	Mutated acetaldehyde-CoA/ alcohol dehydrogenase gene (adh E)	Ethanol tolerance upto 40 g/L	Brown et al. (2011)
		Improvement in growth of organism	
Thermoanaerobacterium saccharolyticum	Deletion of lactate dehydrogenase (Ldh) and phosphotransacetylase (Pta)	Ethanol yield increased 4.2-fold as compared to WT	Argyros et al. (2011)
Clostridium butyricum	Deletion of β-hydroxybutyryl-CoA dehydrogenase (hbd)	Ethanol titer 18-fold higher as compared to WT	Cai et al. (2011)

12.9 Consolidated Bioprocessing of Cellulosics

The consolidated bioprocessing (CBP) offers cost effective and efficient way to produce bioethanol. CBP involves enzyme production, substrate hydrolysis and fermentation in a single reactor. The main challenges in CBP technology include, (i) high enzyme production levels, without affecting ethanol fermentation capacity and (ii) fermentation of hexose and pentose sugars simultaneously, and (iii) tolerating harsh conditions such as high levels of the ethanol and other co-products. Till date no natural organism is known to possess all the features desired for CBP. There are two strategies to develop CBP-enabled microorganisms, which include recombinant production of cellulolytic microorganisms for improved bioethanol yield and engineering of non-cellulolytic organisms to be cellulolytic.

Fusarium, Rhizopus, Aspergillus, Neocallimastix and *Trichoderma* are some fungi that have been reported to possess the ability to convert cellulose to ethanol. They are possess two biological synthesis systems; one for producing cellulases for degrading cellulose to soluble sugars and the second to produce ethanol and other byproducts. The high levels of ethanol produced during fermentation can alter the cell membrane, resulting in inhibition of cell growth. Conventional method of strain improvement by modifying the genes involved in ethanol tolerance and knocking out the genes responsible for the production of byproducts has been proved useful for CBP.

Reconstructing a mini-cellulosome on the yeast and other non-cellulolytic organism's cell surface is another strategy, which facilitates cellulose conversion by the yeast. The display of a surface mini-cellulosome was engineered with cellulases cloned from *Clostridium cellulolyticum.* A significant increase in cellulase activity with an ethanol titer of 1.41 g/L was achieved (Fan et al. 2012). Cellulases, xylanases, and amylases were expressed on the cell surface of different *S. cerevisiae* strains. The fermentation of cellulose, raw starch, and birchwood xylan by recombinant strains exhibit ethanol yields of 0.45, 0.44 and 0.3 g/g substrate, respectively (Kondo et al. 2002).

It is anticipated that this technology will eliminate various limitations involved in other conventional technologies of bioethanol production. Success in this endeavor could lead to a revolution with respect to the low-cost conversion of lignocellulosics to ethanol.

12.10 Pilot Scale Bioethanol Production Plants

Cellulosic ethanol is renewable, clean and doesn't create a food-versus-fuel scenario because it is derived from the abundant organic materials on earth. These attributes have kept researchers and companies focused on developing this biofuel despite the challenges. Companies that are involved in building refineries are Iogen, POET, and Abengoa while companies involved in producing enzymes to degrade lignocellulosics are Novozymes, Dyadic, DuPont and Diversa. World's largest cellulosic ethanol plant [30 million gallon/year (Mgy)] was constructed in US near

Nevada by DuPont in 2015. This technology was licensed by DuPont for a second plant in partnership with Chinese ethanol producer New Tianlong Industry Company in China. Quad County Corn Processors, a 35 Mgy corn ethanol plant in Iowa, has the capacity to produce 2 Mgy of cellulosic ethanol from corn kernel fiber. Pacific Ethanol is the ethanol plant in California, which also produces cellulosic ethanol from corn kernel fiber. The National Renewable Energy Laboratory (NREL) is the US Department of Energy, which has been working on cellulosic ethanol since 1980. They are involved in reducing the cost of enzymes by bioengineering more effective enzymes that will accelerate the process. NREL has reduced the cost of enzymes to produce cellulosic ethanol from $3 a gallon to 30 cents, primarily by bioengineering better enzymes. As per recent data, corn ethanol costs $1.92 a gallon. In 2011, an Italy based Mossiand Ghisolfi Group established the bioethanol plant having 13 Mgy cellulosic ethanol facility. The plant uses a variety of locally sourced feedstock such as wheat straw and *Arundodonax* (a gaint cane). Several plants have been constructed till date in various countries like Australia, Brazil, Canada, China, Denmark, Germany, Italy, India, Japan, Russia, Spain, and U.S. Cellulosic ethanol plants worldwide are listed in Table 12.8.

Praj is a global process solutions company that offers comprehensive solutions to the ethanol industry, is now in the process of installing a second generation cellulosic demonstration plant in India which will lead to commercialization of its in-house developed technology. India's first 2G ethanol plant with a capacity of 10 ton per day has been inaugurated in April 2016 at India Glycols Ltd., Kashipur (Uttarakhand). The plant has been developed by DBT-ICT Centre for Energy Biosciences at ICT Mumbai with the support of the Department of Biotechnology, Ministry of Science

Table 12.8 Cellulosic ethanol plants worldwide

Company	Location	Feedstock
India Glycols Ltd.	Kashipur, India	Various agricultural residue
Hindustan Petroleum Corp. (HPCL)	Bathinda, India	Straws-rice, wheat, maize
Abengoa Bioenergy	Kansas, U.S.	Wheat straw
BlueFire Ethanol	California, U.S.	Multiple sources
DuPont	Tennessee, U.S.	Corn cobs, switchgrass
DuPont	Iowa, U.S.	Cornstover
American Process, Inc	Michigan, U.S.	Wood residues
Mascoma	Michigan, U.S.	Wood residues
POET-DSM Advanced Biofuels	Iowa, U.S.	Corn cobs, husks
SunOpta	Minnesota, U.S.	Wood chips
Xethanol	Florida, U.S	Citrus peels
Inbicon	Zealand, Denmark	Wheat straw
China Resources Alcohol Corporation	China	Corn Stover
Ethtec	Australia	Wood residues
Iogen Corporation	Ontario, Canada	Agricultural, organic residues
Mossi and Ghisolfi Group	Crescentino, Italy	Wheat straw, Arundo donax
Abengoa Bioenergy	Seville, Spain	Wheat, barley, corn, sorghum

and Technology and its public sector undertaking (PSU)-Biotechnology Industry Research Assistance Council (BIRAC). Another ethanol plant has been announced recently by Hindustan Petroleum Corporation Ltd. (HPCL) at Bhatinda, Punjab. HPCL has teamed with International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Engineers India Ltd. (EIL) and Department of Biotechnology (DBT) for this venture. The plant will have the capacity of 100,000 litres per day at the cost of $92.4 million. It will use 400 metric tons of sugarcane bagasse and other crop residues as the feedstock for the production of 3.20 crore litres of ethanol per annum. HPCL and other oil PSUs are setting up 12 ethanol bio-refineries across 11 states (Punjab, Haryana, Uttar Pradesh, Madhya Pradesh, Bihar, Assam, Odisha, Gujarat, Maharashtra, Karnataka and Andhra Pradesh) at an estimated cost of ₹10,000 crores which will produce approx. 35–40 crore litres of ethanol annually. Among the bio-refineries planned are those at Dahej in Gujar, Panipat in Haryana, Bina in Madhya Pradesh and Bargarh in Odisha.

12.11 Cellulosic Biorefinery

A biorefinery is a conceptual model for future biofuel production that converts lignocellulosic biomass to fuels as well as other high value co-products with a zero waste approach. Biorefineries utilize a variety of feedstocks and a larger range of processing technologies. Biorefineries are categorized based on sugar (biochemical), syngas (thermochemical) and biogas platforms depending on raw materials, technological processes and products used. In a typical lignocellulose refinery, the isolated sugars from lignocellulosic biomass are used to produce cellulosic ethanol or biobased products and the residual cellulose and lignin, are utilized in steam and electricity generation. Hemicellulose has been used as the plant gum for adhesives, thickeners, protective colloids, stabilizers and emulsifiers (Kamm and Kamm 2004). Xylose can be fermented to a sweetener (xylitol), which is a sucrose replacement for diabetic patients (Peldyak and Makinen 2002). By integrating production of higher value bioproducts into biorefinery's fuel, overall profitability and productivity of all energy related products can be improved. In 2012, the total amount of feedstock available for biorefineries was 341 million tons, of which 70% was agricultural residues and 30% was forest residues (Balan 2014).

American Process Inc. (an American company) specializes in the development of technologies for the commercial production of sugars and ethanol from biomass. The company has developed three technologies (AVAP®, Green Box+® and Green Power+®) for producing low cost cellulosic sugars and ethanol from non-food based biomass. AVAP® (American Value Added Pulping) technology is a patented biorefinery process. This technology is used to fractionate biomass into its primary components, cellulose, hemicelluloses and lignin. The hemicellulose and cellulosic fractions are hydrolyzed to sugars. Lignin is recovered for use in lignin derived products or can be used in the production of boiler fuel to produce the energy to run the process. GreenBox+® technology allows the extraction of hemicelluloses from wood chips to make a chemical free and high yield corrugated medium pulp. The

technology is currently being demonstrated at the Thomaston Biorefinery in Thomaston, Georgia. GreenPower+®technology increases ethanol output by converting hemicellulose fraction of the biomass to ethanol, while still allowing the rest of the biomass to produce pellets, pulp and power. The technology is currently being demonstrated at the Alpena Biorefinery in Alpena, Michigan. There are various ethanol pilot plants, which are adopting the biorefinery approach (as listed in Table 12.8). Biorefineries are, therefore, seen as a very promising route to meet the desired aims in bioethanol production that allows utilization of each component of biomass into useful products, while preserving the environment.

12.12 Conclusions

Due to increasing energy demand, energy cost and global warming, there is an urgent need to find alternative options. In this regard, lignocellulosic biomass holds significant potential and is gaining importance because of its availability and low price as compared to food based materials like sugar and starch. Successful blend and expression of cellulases, hemicellulases, ligninases and other accessory proteins are required for synergetic action. The challenge has been in the development of successful mix of these enzymes which functions optimally even under different conditions to completely degrade lignocellulosic biomass to monomeric sugars. In current scenario, research is being directed towards genetic engineering for developing improved processes and products. A significant progress has been made in recent years to make biomass less recalcitrant by developing various pretreatment methods, generating recombinant strains to enhance their metabolic product yields and product/inhibitor tolerance, development of various immobilization and fermentation techniques and improved enzyme cocktails to make lignocellulosic biomass derived ethanol commercially viable and profitable. Techniques such as directed evolution and rational design of enzymes are being attempted for improving cellulases. Continued and concerted research efforts are, therefore, called for bringing down the cost of bioethanol.

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Importance of Actinobacteria for Bioremediation

13

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Abstract

A Bioremediation process includes bioinjection, bioaugmentation and phytoremediation and relies on the management of suitable plants and soil microorganisms, either natural or tailor made, that can degrade, sequester, or remove environmental contaminants and are capable of catabolizing the contaminants. Actinobacteria are present in high concentration in soil and use humic acid that helps in recycling substances in nature. They are nonmotile, filamentous, anaerobic bacteria, belonging to order Actinomycetales, present in soil, having amazing metabolic versatility and mechanism to clean up the hazardous pollutants. Actinobacteria are also present under extreme conditions such as high temperature, low moisture, nutrient starvation and producing biosurfactants and speeding up the reaction for biological oxidation and biodegradation of the pollutants. These bacteria have resistance mechanisms for producing as superoxide dismutases, efflux transporters and metal binding proteins. They cause degradation of herbicides, pesticides (organochlorine-lindane), heavy metals, chromium (VI), petrochemicals, nitroaromatics, 2,4,6 trinitrotoluene (TNT), tannery wastes and aromatic compounds. Bioremediation, an appropriate remedy, depends on the nature of contaminants. If the contaminants are susceptible to biodegradation by the site organisms (without the need for human intervention) is known as intrinsic bioremediation. Alternatively, relevant organisms can be added i.e. Engineered bioremediation. In both conditions rate of bioremediation depends on, the type and concentration of contaminant, the microbial community, and the subsurface hydro- geochemical conditions. New niches and extreme micro ecosystems, in terms of temperature, salt concentration, and pH, should be explored to identify and locate new microorganisms able to deal with heavy metals, hydro-

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carbons, chlorinated compounds and in general, all pollutants affecting soil and water. Therefore bioremediation is useful for large scale application on heterogeneous environment such as ground water, soil sludge and industrial wastes.

Keywords
Biosurfactants · Biodegradation · Biosorption · Bioaccumulation · TNT · Remediation · Xenobiotics

13.1 Environmental Pollution and Remediation: Global Scenario

Actinomycetes are prokaryotes, which are biologically important and most economically. They have various habitats like terrestrial, aquatic and extreme climatic conditions. They are gram positive with high G + C (>55%) content in their DNA. They are free living, saprophytic bacteria comprising 10% of the total bacteria colonizing marine aggregates (Valli et al. 2012). *Actinoplanes, Micromonospora, Rhodococcus, Streptomyces, Thermactinomyces* are fresh water actinobacteria. *Dietzia, Rhodococcus, Streptomyces, Salinispora, Marinophilius, Salinibacterium Aeromicrobium marinum* and *A. verrucissipora* are marine actionbacteria. *Streptomyces, Micromonospora, Nocardiaform* and *Strptoverticillum* are terrestrial actinobacteria. Actinomycetes are used for degradation or removal of environmental pollutants; they provide an economic and safe biological method than other physiochemical ones. They can metabolize the contaminants for their growth and reproduction. Waste disposal of pollutants has created insufficiency of clean water and instability of soils, thus diminishing crop production and creating pollution. The main cause of pollution is population explosion that is responsible for decrease in natural resources, rapid expansion of industries, intensive agricultural practices, poor health care, vehicles, etc. All theses reduce the quality of life and cause different types of pollution as shown in Table 13.1. There are various microorganismsin nature that constantly breakdown organic compounds. Pollutants provide a source of carbon, which works as building blocks for the new cell constituents and to produce more cells. They provide electrons, which can obtain energy by breaking chemical bonds and transferring electrons to electron acceptor as oxygen. Actinomycetes possess many properties that make them best candidates for application in bioremediation of soils and recycling of organic carbon. They degrade the organic pollutants (complex polymers) into simple polymers.

Table 13.1 Different type of environmental pollution and their control

Category	Sources	Types of pollutants	Control measures	Major symptoms
Air Polluti ccxvon	Agriculture, mining, industries combustion –engine, vehicle etc	Suspended particulate matter (SPM) as diesel exhaust, acid mist, Gaseous as CO, SO_2, SO_3, NO_2, Hydrocarbons Volatile Organic Compounds (VOC), Secondary Pollutants as O_3, Peroxyacetyl nitrate (PAN)	Condensation, absorption, adsorption, incineration, Wet scrubbers, electrostatic precipitators, filters	Respiratory diseases as asthma, bronchitis, skin diseases, anxiety, irritation to eyes, nose, throat
Water Pollution	Industrial waste, sewage, mining activities, marine dumping, accidental oil leakage, burning of fossil fuels, **c**hemical fertilizers and pesticides	Degradable as domestic sewage, plant nutrients, bacteria and viruses nondegradable as toxic trace metals and radioactive compounds, global warming, radioactive wastes	Ion exchange reverse osmosis, precipitation coagulation, 4R concept (recycling, renovation, recharge and reuse)	Cholera, amoebiasis (traveller's diarrhea), lead poisoning, malaria, arsenicosis
Soil Pollution	Industries, agricultural activities, waste disposal, accidental oil spills, acid rain	Industries as pulp and paper, tanneries, textiles, fertilizers, pesticides, urban wastes as plastics, glasses, metallic cans, fibres, paper, rubbers radio nuclides of radium, thorium, uranium, large amount of human, animal and bird excreta	Planting trees, contour cultivation and strip cropping proper dumping of unwanted materials, decreased soil fertility alteration in soil structure, reducing the use of chemical fertilizer and pesticides, recycling paper, plastics, avoiding deforestation and promoting forestation	Congenital illnesses and chronic health diseases.

(continued)

Table 13.1 (continued)

Category	Sources	Types of pollutants	Control measures	Major symptoms
Noise Pollution	Industrialization social events, transportation, construction activities, household chores and urbanization	Industry machines (various factories, industries and mills) musical instruments, pyrotechnics (shooting off fireworks and firecrackers)	neurological problems, birth defects and abortion, making skin pale, excessive adrenalin in the blood stream high blood pressure, mental distress, heart attacks	Psychiatric illness, psychological and pathological disorders
Ground Subsidence	Up swelling of groundwater, circular surface pits under the earth, coal mining	Dissolution of soluble rocks, such as limestone, beneath the earth surface, the thawing of frozen ground	Structural damage to building	Sink holes can be a result of this dissolution, earthquakes volcanoes, and climate change
Thermal pollution	Nuclear Power plant, coal fore, industrial power plant, domestic sewage, hydroelectric power plant		Wet Cooling towers, dry Cooling towers, cooling ponds, spray ponds	Ecosystem disbalance, decrease dissolved oxygen, eutrophic conditions, unexpected migration of water animals

The land, air, water resources site have been contaminated due to rapid industrialization causing pollutants, threatening humans and quality of local, regional and global ecosystems with serious health risks. For controlling different types of pollution, two specific approaches are proposed:

(i) **Assimilative capacity concept** the principle of this asseverates the existence of a specified level of emissions into the environment leading to unacceptable environmental or human health effects
(ii) **Principle of control concept,** which avoids the environment (air, water and soil) damages by controlling the manner, time and rate at which pollutants enter in the environment by using end-of-pipe filters and scrubbers and primarily directed at point source discharges.

The wastes from industries and sewage contain different organic and inorganic chemicals as heavy metals, salts and extreme pH. The cumulative effect of these chemicals on long term basis has detrimental serious effects on human health. Bioremediation is the technique to clean the environment and its resources by

completely destroying the pollutants or to transform them into some biodegradable substances. It is the naturally occurring process in which the microorganisms or plants either immobilize or transform environmental contaminants to innocuous state end products (Mueller 1996). This is an environment friendly technology, with low cost and safer with high efficiency. It has selectivity to specific metals requiring no additional nutrient with enhancement of metal recovery and regeneration of biosorbent. It can be carried out at any site (Van Dillewijn et al. 2007; Kratochvil and Volesky 1998). It included burning, catalytic devastation, the use of adsorbents and the physical elimination which help in reducing the pollutants. Bioremediation processes depend on many factors as microbes (biomass concentration, population diversity, aerobic/anaerobic growth, enrichment of the capable microbial populations, production of toxic metabolites, lack of nutrients), physico-chemical bioavailability of pollutants (equilibrium sorption, irreversible sorption, incorporation into humic matters), the contaminants (chemical structure of contaminants, toxicity of contaminants, solubility of contaminants), and the environmental factors (depletion of preferential substrates, electron acceptor/donor, oxygen content, 5.5–8.8 pH, type of nutrients C,N,P, soil type, low clay or silt content, 15–45 °C temperature, 45–85% moisture content, 25–28% water holding capacity, mass transfer limitations (oxygen diffusion and solubility, diffusion of nutrients, solubility in/miscibility with water). First patent was registered by using *Pseudomonas putida* in 1974 to degrade the petroleum compounds (Yadav and Loper 2000). Different bioremediation agents as microbiological cultures, enzymes and nutrient additives increase the rate of biodegradation to mitigate the effects of pollutants by U.S. EPA. Different bioremediation techniques with advantages and disadvantages are shown in Table 13.2.

13.2 Bioremediation of Organic and Metal Contaminated Environment

Generally metal contaminants are not biodegradable. Microorganisms reacting/acting upon metals lead to the establishment of a tolerant or resistant microbial population by this inhibit biodegradation. Bioremediation is related to the composition, complexity, physiological and ecological components, species, solubility and concentration of metals and the characteristics of the influent, such as pH, as well as presence and concentration of other cations and/or molecules, and suspended solids (Gikas 2008). Metal contaminants accumulation cause specific toxicity symptoms at higher concentration, harmful effects on human, animal health and ecological damage (Meena et al. 2005) by altering the conformation structure of nucleic acids, proteins and interference with oxidative phosphorylation (Yaoa et al. 2008). Metal-rich mine tailings, electroplating, gas exhaust, energy and fuel production, weathering of minerals, erosion, volcanic activity, downwash from power lines, intensive agriculture use of pesticides, phosphate fertilizer discharge, biosolids (e.g., livestock manures, composts, and municipal sewage sludge), and sludge dumping are the sources of heavy metals in soils and aqueous streams as both natural

Table 13.2 List of techniques used in bioremediation of contaminants

Remediation techniques	Advantages	Disadvantages	References
Natural attenuation (Intrinsic bioremediation) uses the indigenous microorganisms. It limits the flow of contaminants and also reduces their concentration at contaminated sites without any remediation wastes.	Remediation waste is least which has less impact act on the environment and without human intervention	Longer periods of time may be required.	Ebuehi et al. (2005)
	Can be easily combined with other technologies.	Costly and complex site characterization.	
Phytoremediation uses plants in combination with microorganisms to help remediate the contaminated site.	Least environmental disturbance.	Limited to soils less than 1meter from the surface and ground water less than 3 meter from the surface	Marchiol et al. (2007)
	Solar energy driven technology.		
Plants help in accumulation, removal or conversion of pollutants.	Used on a wide range of contaminants	Contaminants may enter the food chain through animals which eat the plants used.	
	Cost-effective for large contaminated sites		
Biosparging *In situ* technology. Used in environment for uniform permeable soil. Air and nutrients are injected into the saturated zone to increase the biological activity of the indigenous microorganisms.	Readily available equipment.	Biochemical and physiological interactions are very complex and not understood.	Raag (2000)
	Cost competitive.	Migration of constituents can lead to toxicity elsewhere.	
	Allows substantial flexibility in the design and structure of the arrangement.		
Bioventing *In-situ* process combines an increased oxygen supply with extraction of vapour and minimize or eliminate the off-gassing of volatilized contaminants to the atmosphere.	Very economic and easy to install	High concentrations can be toxic for microorganisms	Mihopoulos et al. (2002)
	Any aerobically degradable compounds contained by the soil	Low soil permeability doesn't allow proper implication.	
	Can be combined with other technologies.	Good for unsaturated zones of soils.	

Bioaugmentation used for soil and groundwater involves addition of highly concentrated and specialized populations of specific microbes into a contaminated site to enhance the rate of contaminant biodegradation as chlorinated ethenes.	Related to its chemical properties, as well as to a broad range of soil physical and chemical parameter.	Used in community wastewater handling.	Lee et al. (2006)
	Normal microbial strains or genetically engineered microbes or both.	Microbes generate own waste products.	
		Competition between the introduced species and the bioavailability of the xenobiotic compounds.	
Bioreactors/Bioslurry used for remediation of soil contaminated with explosives.	Fast degradation.	Very high contaminant concentrations may be toxic to microorganisms.	Zhang et al. (2001)
	Effective use of inoculants and surfactant.	Expensive.	
Composting uses cow manure and mixed vegetable waste to remove the toxicants upto 90% from the contaminated soil.	Volume and moisture content of the waste reduces.	Treatment time more than other techniques	Atagana (2004)
	Destruction of pathogens and odor producing nitrogen- and sulfur-containing compounds.	Requires nitrogen supplementation.	
		Volatile contaminants may be released during composting.	
Biopiling involves for petroleum-contaminated soils into piles or heaps and then simulating aerobic microbial activity by aeration and the addition of minerals, nutrients and moisture.	Full-scale capability.	Excavation of contaminated soils is required.	Filler et al. (2001)
	Aeration is provided by pulling air through the pile with a vacuum push.	Mass transfer problem.	
		Treatment time is characteristically 3 to 6 month.	
	Moisture, heat, nutrients, oxygen, and pH are controlled to enhance biodegradation	An electron acceptor is required.	
Land Farming bioremediation process that is performed in the upper soil zone or in biotreatment cells.	Relatively simple in design and implementation.	Required area is large	Hejazi (2002)
	Short treatment times.	Dust and vapor generation may cause some air pollution.	
Bioslurping combines elements of bioventing and vacuum-enhanced pumping for remediation of the contaminated site.	Applied at shallow as well as deep sites.	Low soil permeability hampers remediation.	Yen et al. (2003)
	Recovers free product, thus speeding remediation	Soil moisture and oxygen content limits the microbial activities.	
		Low temperatures slow remediation.	

Table 13.3 Sources of heavy metals in the environment

Heavy metals	
Natural sources	Anthropogenic sources
Weathering of minerals	Pesticides, wood preservatives, biosolids, ore mining and smelting
Erosion and volcanic activities	Cd: Paints and pigments, plastic stabilizers, electroplating, phosphate fertilizers
Biosolids (e.g., livestock manures, composts, and municipal sewage sludge)	Cr: Tanneries, steel industries, fly ash
Forest fires and biogenic source	Cu: Pesticides, fertilizers, ore mining and smelting
	Hg: Mining for Au-Ag, coal combustion, medical waste
Particles released by vegetation	Ni: Effluent, kitchen appliances, surgical instruments, automobile batteries
	Pb: Aerial emission from combustion of leaded fuel, batteries waste, insecticide and herbicides.

Modified after Dixit et al. (2015)

components and result of human activity (Raskin et al. 1994). Several metals are required for biological system at a certain concentration as shown in Table 13.3 (Collins and Stotzky 1989). Some metals such as mercury, lead, cadmium and chromium are cytotoxic and mutagenic as well as carcinogenic. Their toxic effect on kidneys, nervous system, may lead to the symptoms of mental disorder, weakness, headaches, abdominal cramps, diarrhea, and anemiain certain cases even permanent damage of the organelles. Even though copper, nickel, cobalt and zinc are not toxic at low concentration, still due to extensive use their aggregate level is high in the environment, leading to a serious concern for health of global population (Wang et al. 2009).

Pesticides such as hexacholorobenzene (HCB), hexachlorocyclohexane isomers (HCHs), dichlorodipheny tetrachloride (DDTs) are the major pollutant. Urban areas present statistically significant higher levels for all families studied and HCB, at remote locations revealing anthropogenic activities as potential sources for HCHs and DDTs (Torre et al. 2016). *Streptomyces* sp. bioremediated heavy metals from waste water and percent removal of this as Cd^{+2}12, Cr^{+2} 22, Cu^{+2} 16, Fe^{+2} 24, Ni^{+2} 12, Zn^{+2} 11, Mn^{+2} 79 and Pb^{+2} 32 (Majdah and Ahmed 2016).

Mechanism for bioremediation Heavy metals are bioremediated by changes of oxidation through the microbial pathways. Microorganisms are capable of two-way defense (i) production of degradative enzymes for the target pollutants and (ii) resistance to relevant heavy metals. It occurs by two ways:

(i) **Direct reduction** It is an ex-situ method by using metal reductase enzyme. This method has low efficiencies for metal extraction. It is applicable for groundwater decontamination, using bioreactors and can also be done for soils after excavation (pulping or heaping and inoculation with appropriate microbial consortium).

(ii) **Indirect reduction** It is an in-situ method by using sulfate reducing bacteria to react and precipitate the metals. It is an environmentally sound and inexpensive alternative to pump and treat contaminated groundwater or excavate and treat the contaminated soils. Microbial growth is induced in sub-surface zones by injecting substrates. The migrating metals are intercepted and immobilized by precipitation with biologically produced H_2S.

There are three microbial processes for bioremediation of metals by actinomycetes as follows:

(i) **Biosorption and Bioaccumulation** Biosorption is sequestration of the positively charged heavy metal ions (cations) with the negatively charged microbial cell membranes. Bacterial polysaccharides secreted as slime on their outer surface for capsule formation. From the surface, the metals are transported into the cell cytoplasm through the cell surface with the help of transporter proteins and get bioaccumulated.
(ii) **Biologically Catalyzed Immobilization** Inside the microbial cells, metal ions gets fixed as Iron (Fe)-oxides and form organic colloids which become immobilized by enzymatic reduction.
(iii) **Biologically Catalyzed Solubilization** Metal reducing bacteria enzymatically reduce and under appropriate conditions, solubilize oxide minerals. Such dissolution has been shown to release cadmium (Cd), nickel (Ni) and zinc (Zn) into solution during reduction of goethite (a form of Fe-oxide) by anaerobic bacterium, *Clostridium* spp.

Actinobacteria can detoxify metals by transformation of valence, extracellular chemical precipitation or volatilization. They change the valance states of metals by converting the contaminants into immobile or less toxic forms. Actinobacteria as *Thiobacillus* sp. (sulphate reducing bacteria) is used to treat the metal leachates (Macaskie et al. 2005). Microbial response to heavy metals depends on different factors such as the concentration and availability of the metals, nature and type of medium (Fig. 13.1). Microorganisms develop different detoxifying mechanisms for survival in metal polluted habitats in both *ex-situ* and *in-situ* as biosorption, bioaccumulation, biotransformation and biomineralization. In bioaccumulation heavy metals uptake actively while passively in adsorption. Some processes for waste treatment through biosorption require the addition of nutrients for enhancing oxygen demands as biological oxygen demand (BOD) or chemical oxygen demand (COD).

In non-chlorinated organic, biodegradation is enhanced by aerobic degradation upon supplying O_2 and inorganic nutrients but it does not limit the rate of contamination. Nitrate and Fe(III) have been considered as electron acceptor for bioremediation of petroleum-related ground water contaminants (Macy et al. 1993).

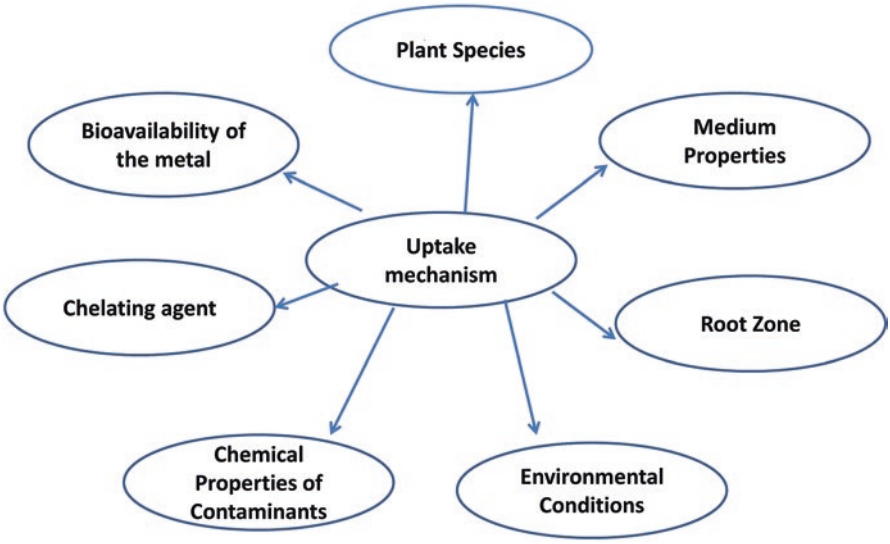

Fig. 13.1 Uptake mechanisms of heavy metals

A mass balance of heavy metals in the soil environment can be expressed using the formula:

$$\mathrm{M}total = \left(\mathrm{M}p + \mathrm{M}a + \mathrm{M}f + \mathrm{M}ag + \mathrm{M}ow + \mathrm{M}ip\right) - \left(\mathrm{M}cr + \mathrm{M}_l\right) \qquad (13.1)$$

where M is the heavy metal, *p* is the parent material, *a* is atmospheric deposition, *f* is fertilizer source, *ag* is agrochemical source, *ow* is organic waste source, *ip* is inorganic pollutant, *cr* is crop removal and *l* is losses by leaching, volatilization and other processes.

It is estimated that emission of several heavy metals in viz atmosphere from anthropogenic sources is three times higher than the natural sources (Sposito and Page 1984). For example.

(i) **Microbial reduction of Chromium (VI) to Cr (III)** World production of chromium is of the order of 10,000,000 tons per year in industries as in leather and tannery industries, electroplating, steel and automobile manufacturing, production of paint pigments and dyes, refractory and in wood preservation. It exists in several oxidation states, ranging from Cr(II) to Cr(VI), but in soils the most stable & common forms are trivalent Cr(III) and hexavalent Cr(VI) species. The trivalent & hexavalent forms can inter-convert. Chromium tolerance, resistance and reducing ability occurs in group of microorganisms as viz *Arthrobacter crystallopoites* (500 mg/L), *Pseudomonas sp*. CRB 5 (520 mg/L), *Bacillus maroccanus* Chr A21 (1040 mg/L), *Corynebacterium hoagii Chr* B20 (1144 mg/L), *Bacillus cereus* ES04 (1500 mg/L) (Viti et al. 2003). Anaerobic sulfate reducing and methanogenic bacteria possess inherent abilities to absorb more than 90% of chromium to its cell biomass.

Table 13.4 Selected plants for phytoremediation of heavy metals

Heavy metal	Plant species	Uptake mechanisms and media (substrate)	References
As	*Leersia oryzoides* (rice-cut grass) *Brassica junceavar*. Varuna and Pusa Bold—terrestrial	Phytoextraction (soil was added to aqueous solution)	Ampiah-Bonney et al. (2007)
Pb	Creeping zinnia (*Alternanthera phyloxeroides*) Moss rose (*Sanvitalia procumbens*) Alligator weed (*Portulaca grandiflora*)	Phytoextraction (soil mixture and Fly ash)	Cho-Ruk et al. (2006)
Zn	Eastern gamagrass (*Tripsacum dactyloides*)—terrestrial	Soil (agricultural land)	Hinchman et al. (2000)
Cd and Zn	Willow (*Salix viminalis*), *Raphanus sativus L.*	Phytoextraction (soil agricultural land)	Hamon et al. (1999)
Pb, Zn, Ni	*Scirpus littoralis*	Laboratory pot experiment (Fly ash and soil mixtures)	Bhattacharya et al. (2006)
Cu, Cd, Cr, Zn, Fe, Ni, Mn, and Pb	Wheat (*Triticum aestivum L*) Indian mustard (*Brassica campestris L.*)	Field experiment (Soil agricultural land area)	Chandra et al. (2009)
Cd, Cr, Cu, Ni, Pb, and Zn	*Brassica juncea* (Indian mustard), *Brassica rapa* (field mustard), and *Brassica napus* (rape)—terrestrial	Soil	Van Ginneken et al. (2007)
Cd, Pb, Zn	Corn (*Zea mays*)	Phytoextraction (soil mixture)	Amin (2011)

(ii) **Microbial reduction of Uranium (VI) to U(IV)** Uranium is present in uranyl carbonate in groundwater and microbes convert it to highly insoluble U(IV). *Geobacter sp.* removes uranium (U) from the groundwater (Anderson et al. 2003).

(iii) **Microbial reduction of Selenium (VI) to elemental Se (0)** causes precipitation of the metal selenium and reduced bioavailability. In addition, SeO_4 can be microbial methylated to volatile dimethyl selenide which easily escapes from soil.

Bioremediation of Heavy Metals by Plants Growing green plants helps to clean up hazardous waste from the contaminated sites is known as phytoremediation (Raskin and Ensley 2000). It can be used as intrinsic as well as extrinsic application. Intrinsic bioremediation is also known as green technology as it minimizes the disturbance of the environment and also reduces the spread of contaminants in environment (Table 13.4). The mechanisms of bioremediation by plants include:

1. **Phytoextraction** Plants absorb the concentrated metals by producing the biomass. They have the capability to extract large concentrations of heavy metals through roots and translocate them to the surface. Different factors are responsible for the process such as: metal bioavailablity within the rhizosphere, metal uptake, metal fixed within the roots, xylem loading/translocation to shoots, metal tolerance (Camargo et al. 2003).
2. **Phytostabilization** occurs for metals like arsenic, cadmium, chromium, copper and zinc (Kunito et al. 2001). Plant roots remediate the contaminants through mobility and bioavailability process. In this the plants decrease the water percolation through the soil matrix, and act as a barrier to prevent the direct contact with contaminated soil. It occurs through the sorption, precipitation, complication or metal valance reduction. This process has some disadvantages as contaminants remain in the soil and need extensive fertilizer application.
3. **Rhizofiltration** works for metals like Pb, Cd, Cu, Ni, Zn, and Cr which primarily get accumulated within the roots and get remediated by rhizofiltration. Plants like sunflower, Indian mustard, tobacco, rye, spinach, and corn easily removed lead from water or soil (Camargo et al. 2003). Terrestrial plants having fibrous and elongated root systems are preferred for this process.
4. **Phytovolatilization** Plants take the contaminants from soil, which pass through the xylem vessels towards the leaves and get converted into volatile forms to reach atmosphere through transpiration (Heaton et al. 1998). Mercury is remediated by this process.

13.3 Bioremediation of Xenobiotics and Agricultural Chemicals

Xenobiotics are the anthropogenic compounds that persist in the environment (medium to long-term stability) and their persistence results in significant impact on the soil ecosystem and health problems (Cuthbertson and Murchie 2010).

Direct sources of the principal xenobiotics include pesticides, fuels, solvents, alkenes, polycyclic hydrocarbons (PAHs), synthetic azodyes, pollutants (dioxins and polychlorinated biphenyls), polyaromatic, chlorinated and nitro-aromatic compounds (Sinha et al. 2009). **Indirect sources** of xenobiotics include Non-Steroidal Anti inflammatory Drugs (NSAIDs), pharmaceutical compounds, pesticides residuals. Xenobiotics are degraded by the group of degradative enzymes. They have two major categories, viz. peripheral and ring-cleavage enzymes. Peripheral enzymes such as oxygenase catabolize the pollutants initially to a metabolite and then degrade it, rendering them susceptible to the enzymes of ring-cleavage pathway (Fig. 13.2).

Alexander (1965) proposed microbial infallibility as the process in which microorganisms play an important role in the degradation of naturally occurring compounds. Xenobiotics do not undergo biological transformation but microorganisms as actinobacteria play an important role in degradation of xenobiotics (Table 13.5) and maintain a steady state of chemicals in the environment. Effective microorganisms (EM) form the consortia of naturally occurring microorganisms which secrete

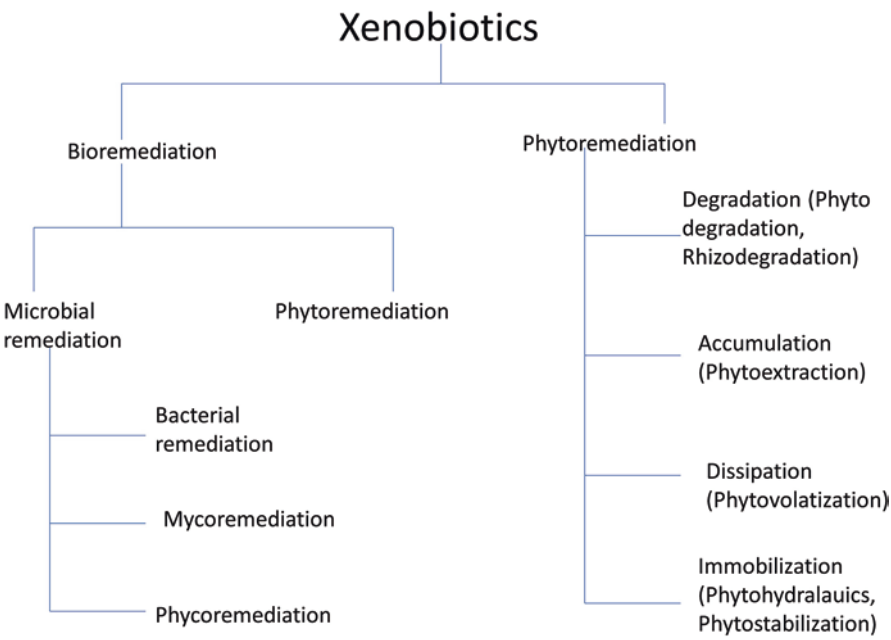

Fig. 13.2 Classification showing organisms involved in microbial and phytoremediation of xenobiotics

the organic acids and different extracellular enzymes, such as laccases, ligninases, peroxidase, aminoacylpropane-1-carboxylase and peroxidases are very important in degradation of xenobiotics.

There are two objectives in relation to biodegradation of xenobiotics:

(i) How biodegradation activity arises, evolves and transferred among the members of soil micro flora, and.
(ii) To device bioremediation methods for removing or detoxifying high concentration of dangerous pesticide residues (Gupta and Mukerji 2001). Following two actions are main modes for degradation of the xenobiotic compounds:
(a) Aerobic biodegradation and (b) Anaerobic biodegradation.

Aerobic biodegradation is used for petroleum hydrocarbons, chlorinated aliphatic, benzene, toluene, phenol, naphthalene, fluorine, pyrene, chloroanilines, pentachlorophenol and dichlorobenzene. The mechanisms for degradation of xenobiotics were proposed by Shimao (2001), Kyrikou and Briassoulis (2007).

$$\text{Xenobiotic compounds} + O_2 \rightarrow CO_2 + H_2O + \text{biomass} + \text{residue(s)}$$

Anaerobic Biodegradation is used by anaerobic sulphate reducing and methanogenic bacteria viz *Acidovorax, Bordetella, Pseudomonas, Sphingomonas, Variovorax, Veillonella alkalescens, Desulfovibrio sp, Desulfuromonas*

Table 13.5 Actinobacteria and xenobiotics degradation

Microorganism	Pollutants	References
Pseudomonas sp	Aliphatic and aromatic hydrocarbons-degradation of oils polychlorinated biphenyls (PCBs), Pesticides, Fungicides, phenols, heavy metals and phenolics, dyes	Wasi et al. (2010) and Joe et al. (2011)
Anoxybacillus pushchinoensis, Anoxibacillus kamchatkensis, A. flavithermus (Thermophilic)	Effluents containing Dyes such as azo dye degradation	Gursahani and Gupta (2011)
Brevibacillus borstelensis and *Rhodococcus ruber*	Polythene used for manufacturing plastics	Hadad et al. (2005)
Mycobacterium sp	Polycyclic aromatic compounds, Benzene, branched hydrocarbons, cycloparaffins	Mrozik et al. (2003)
Alcaligenes sp	Polychlorinated biphenyls, alkyl benzene, halogenated hydrocarbons	Sudip et al. (2002)
Nocardia sp	Naphthalene, alkylbenzenes, phenoxyacetate	Das and Adholeya (2011)
Anthrobacter sp	Benzene, poycyclic aromatics, phenoxyacetate, pentachlorophenol	Das and Adholeya (2011)
Cornebacterium sp	Halogenated hydrocarbons, phenoxyacetate	Jogdand (1995)
Xanthomonas sp	polycyclic hydrocarbons	Jogdand (1995)
Streptomyces sp	Halogenated hydrocarbons, Phenoxyacetate	Jogdand (1995)

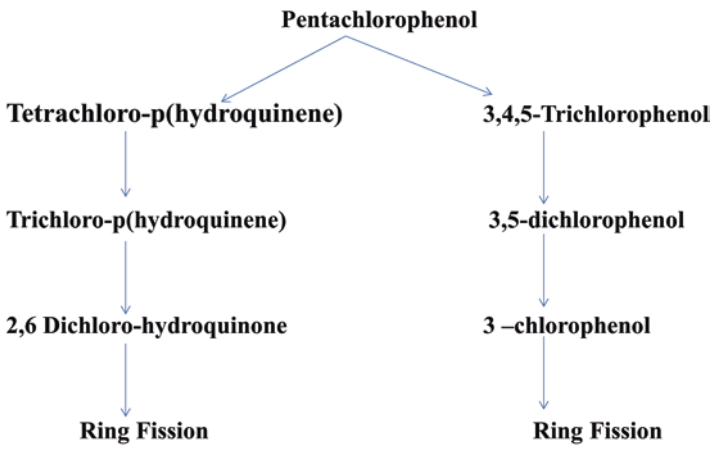

Fig. 13.3 Aerobic and anaerobic biodegradation of Pentachlorophenol

michiganensis, Desulfitobacterium halogenans, Desuluromonas oleovorans, Geobacter metallireducens and *Desulfitobacterium acetonicum* polychlorinated biphenyls (PCBs), chlorinated dioxins and pesticides like DDT get degraded by anaerobic process (Fig. 13.3).

$$\text{Xenobiotic compounds} \rightarrow CO_2 + CH_4 + H_2O + \text{biomass} + \text{residue}(s)$$

Actinobacteria enhance the bioremediation of agricultural chemicals by biosorption, biomineralization, biostimulation, cell immobilization, production of biosurfactants, design of defined mixed cultures and the use of plant-microbe interaction (Analia et al. 2016). Xenobiotics biodegradation by actinobacteria is mainly by biosorption process which requires binding and concentration of selected ions or other molecules from aqueous solutions by biomass or certain biomolecules (Volesky 2007). **Biostimulation** neither involves modification of the environment to stimulate existing bacteria (by the addition of nutrients, oxygen or other electron donors and acceptors to the coordinated site in order to increase the population) nor the activity of naturally occurring microorganisms (Perfumo et al. 2007). **Biomineralization** Archaebacteria has proteinaceous surface layer (S- layer) that works as an interface between the cell and the environment. The protein layers provide numerous functional groups that interact with metals, such as COOH, NH_2, OH, PO_4 and SO_4. S layer also traps ions and/or prevents the uptake of toxic metals into the bacterial cell. It also acts as an ideal barrier against toxic dissolved metal ions. Calcium, strontium, arsenic, and antimony bind with S layer. Binding makes metal complexes with layers as an initial step of bio-mineralization (Phoenix et al. 2005). The S-layers provide crystallization nuclei and serve as a biomineralization template. Wang and Chen (2009) have also discussed the involvement of S-layers in the formation of polymetallic nodules. Actinomycetes decolorize and detoxified azodyes composed of phenylamine, benzenediazonium chloride or phenol by disintegrating azo bonds of the dyes, and formation of colorless amines and simpler compounds (Kumar et al. 2016).

13.4 Bioremediation of Lignins and Polyphenols

Lignin is a heteropolymer containing polyphenolic group made of three phenyl propionic alcohol monomers as p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Agbor et al. 2011). It provides structural support, making the whole structure robust and resistant (Brodeur et al. 2011). It acts as a cementing agent by providing impermeable barrier to enzymes. Therefore lignin is used in synthesis of chemicals (Suhaset et al. 2007) and biocomposites (Le Digabel and Averous 2006) or polymers (Stewart 2008) due to presence of activated carbon and phenol. Lignocelluloses perform important functions in biorefinery processes and for power generation.

Microbial cellulase systems are either complex or noncomplex (Lynd et al. 2002) divided into exoglucanases (EC 3.2.1.74), endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) (Del-Pulgar and Saadeddin 2014). Anaerobic bacteria have complex system known as cellulosomes, multienzyme complex protuberances from cell surface stabilized by dockerin and adhesion proteins. Noncomplex system is characterized by aerobic bacteria including most of the actinobacteria that secrete extracellularly using specific secretion pathways.

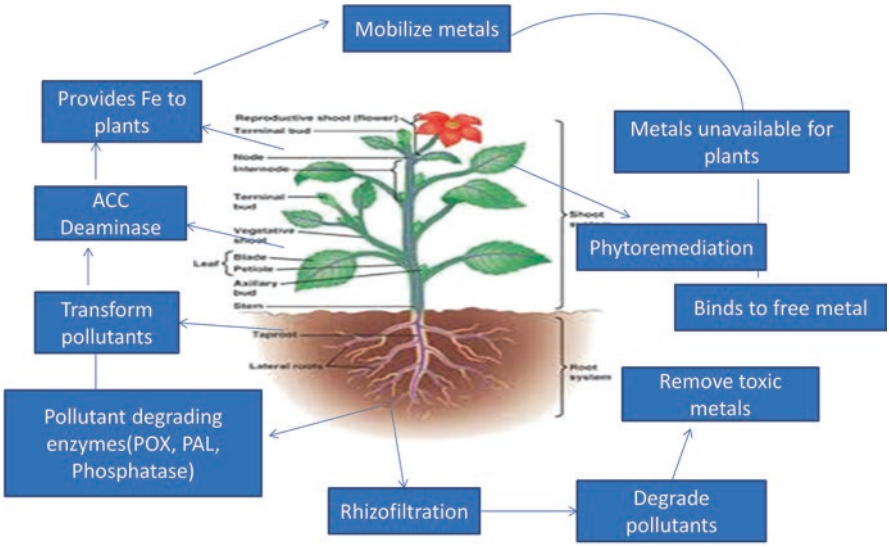

Fig. 13.4 Effect of enzymes on bioremediation (*POX* peroxidases, *ACC* aminoacylpropane-1-carboxylase, *PAL* phenylalanine ammonia lyase)

Actinobacteria as *Cellulomonas fimi, Microbispora bispora* and *Thermobifida fuscahave* are cellulase producing microorganisms (Del-Pulgar and Saadeddin 2014). *Cellulomonas fimi* and *Cellulomonas flavigena* are facultative anaerobes, producing free cellulases (Christopherson et al. 2013). Degradation of lignin is mediated by a complex of enzymes principally containing laccases (EC 1.10.3.2), manganese peroxidases (MnP, EC 1.11.1.13), and lignin peroxidases (LiP, EC 1.11.1.14) (Placido and Capareda 2015) (Fig. 13.4). The effect of different factors for lignin degradation occurs as:

(a) Low nitrogen concentration is required for lignin degradation, and mineralization of lignin occurred during secondary metabolism, (b) 100% oxygen giving the highest mineralization, thus showing lignin degradation to be oxidative, (c) Agitation has detrimental effect on lignin mineralization, and (d) A concomitant production of veratryl alcohol upon lignin degradation.

Lignin and lignocelluloses are degraded by lignin peroxidases, and generate H_2O_2 and other easily diffusible activated oxygen species, such as hydroxyl radicals (OH^-), superoxide anion radicals (O_2^-), and singlet oxygen (O_2) (Table 13.6).

Pencillium chrysosporium produces lignin peroxidases (LiPs) with manganese peroxidases (MnPs). Lignin peroxidase is considered the most important lignin-degrading enzyme. *Streptomyces* are well-known degraders of lignin and can mineralize up to 15% of labeled lignins (Berrocal et al. 1997) and solubilize part of lignin. The end product is water-soluble acid-perceptible polymeric lignin. Degradation of lignin compounds is as follows: Demethylation of aromatic ring structures; oxidation of C-α to introduce a carbonyl group; and α-β cleavage to monomeric products. Peroxidases are secreted by actinomycetes in multiple forms.

Table 13.6 Actinobacteria for lignin and plastic degradation

Plastics degradation	Compound	References
Actinomadura miaoliensis BC 44T-5^T	PHB	Tseng et al. (2009)
Actinomadura keratinilytica T16-1	PLA	Sukkhum et al. (2012)
Thermobifida fusca	Terephthalic acid	Kleeberg et al. (1998)
Thermobifida alba AHK119	Terephthalic acid.	Hu et al. (2010)
Microbispora rosea subsp. *aerata* IFO 14046 *Microbiospora rosea* subsp. aerate IFO 14047 *Excellospora japonica* IFO 144868 *E. viridilutea* JCM 399	PTMH and PCL	Jarerat and Tokiwa (2001)
Streptomyces sp. strain MG	PTMH and PCL	Tokiwa and Calabia (2004)
Streptomyces thermoviolaceus subsp. *thermoviolaceus* 76T-2	PCL	Chua et al. (2013)
Streptomyces bangladeshensis 77T-4	PHB	Hsu et al. (2012)
Dietzia sp. Strain GS-1	Disodium terephthalate	Sugimori et al. (2000)
Degradation of organic pollutants		
Streptomyces setonii strain ATCC 39116	Phenol and benzoate	An et al. (2000)
Pseudonocardia thermophila JCM3095	Acrylonitrile	Yamaki et al. (1997)
Kocuria rosea HN01	DDT	Wu et al. (2014)
Dietzia natronolimnaea JQ-AN	Aniline	Jin et al. (2012)
Georgenia daeguensis	4-Chlorophenol	Woo et al. (2012)
Nocardioides sp	2,4-Dichlorophenol and 2,4,5-trichlorophenol	Maltseva and Oriel (1997)
Dietzia sp. Strain DQ12-45-1b	Petroleum hydrocarbons and crude oils	Wang et al. (2011)
Dietzia cinnamea P4	Petroleum hydrocarbons	Weid et al. (2007)
Dietzia sp.PDA 1	Azo dye	Das et al. (2016)
Dietzia sp. E1	Long chain *n*-alkane	Bihari et al. (2010)
Dietzia sp. *H0B*	Prestige oil spill	Alonso-Gutierrez et al. (2011)
Degradation of *Rubber*		
Streptomyces strain La 7	Latex and natural rubber	Gallert (2000)
Actinomadura nitrigenes, *Nocardia farcinica*, *Thermomonospora curvata*	Poly (cis-1,4 –isoprene)	Ibrahim et al. (2006)

These peroxidase isoforms are isolated from *Streptomyces* strains. Actinomycete extracellular peroxidases attack and depolymerize lignin as shown in Table 13.6. These strains demethylate the lignin and oxidize C-α on the phenyl propane side-chain.

Composting has been demonstrated to be effective in biodegrading polycyclic aromatic hydrocarbons (PAHs) (Canet et al. 2001; Potter et al. 1999), chlorophenols (Laine et al. 1997), polychlorinated biphenyls (Block 1998), explosives (Gray 1999)

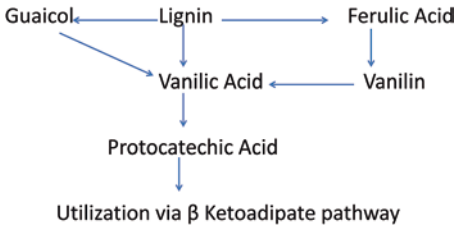

Fig. 13.5 Biochemical pathway for Lignin degradation

and petroleum hydrocarbons (Namkoong et al. 2002) at the laboratory and/or field-scales. Compositing and addition of compost act as stimulants of natural attenuation (Kastner and Miltner 2016). These techniques have relevant physical, chemical and biotic factors and mechanisms for improved contaminant degradation triggered by addition of compost. It is a super bioaugmentaion with a complex natural mixture of microorganisms and also combined with biostimulation by nutrient containing degradable organic substrates that improves the abiotic soil conditions and improving microbial activity (Fig. 13.5).

13.5 Bioremediation of Petroleum Refinery Effluent

Petroleum is a complex mixture of hydrocarbons (benzene, toluene, naphthalene, benzopyrene) and resins. Most of them are stable, toxic and carcinogenic (Yemashova et al. 2007). The components of oily sludge from refineries are extremely complicated includes water, oil emulsion and impurities of suspended solids. The quantitative and qualitative aspects of degradation of petroleum depend on the nature and amount of oil or hydrocarbon present, the ambient or seasonal environmental conditions and the composition of the autochthonous microbial community. Bacteria as well as actinobacteria are promising candidates for microbial based oil recovery. Oil degrading microorganisms are ubiquitous (Table 13.7). Soil contaminated with petroleum is being bioremediated using **natural attenuation processes** (Holden et al. 2002) and **biodegradation**. Bioaugmentation and compositing are good remediation techniques for petroleum refinery effluent. However using actinobacteria is limited due to the deferential effects of conditions on microbial life including disruption of cell membrane, denaturation of enzymes, low solubility of oxygen, low solubility of hydrocarbons, and desiccation (Pernetti and Di Palma 2005) The degradation of crude and refined oils involve a consortium of microorganisms. Actinomycetes like *Pseudomonas sp*. and *Azotobacter vinelandii* as a consortium degraded petroleum hydrocarbons very well ranging between 66.83 and 69.6%. Hydrocarbons get converted into CO_2 and water with release of energy and cell mass that helps microbial growth and activities (Onwurah and Nwuke 2004). *Pseudomonas sp.* degraded diesel by removing long & medium-chain alkanes (Ghazali et al. 2004). *Actinomadura sp, Brevibacillus sp*. and uncultured bacterium clone as a microbial consortium enhanced oil recovery for biopolymer production in different treatment processes developed by Jing et al. (2008).

Table 13.7 Actinobacteria capable of degrading petroleum hydrocarbons

Compound	Microorganisms	References
Alkanes	*Acinetobacter calcoaceticus*	Lal and Khanna (1996)
	Nocardia erythropolis	Hua et al. (2003)
	Pseudomonas sp	Herman et al. (1997)
Mono-Aromatic hydrocarbons	*Pseudomonas sp.*	Churchill et al. (1995)
	Sphingomonas paucimobolis	Willumsen and Arvin (1999)
Poly-Aromatic hydrocarbons	*Achromobacter sp., Mycobacterium sp. Pseudomonas sp. Mycobacterium flavescens, Rhodococcus sp.*	Sunggyu (2003), Doong and Lei (2003), Volkering et al. (1993), Straube et al. (1999), Kwok and Loh (2003), and Churchill et al. (1995)

Franzetti et al. (2008) summarized by stating that bioremediation is an economical tool for management of sites contaminated with organic pollutants. Whang et al. (2008) identified bioremediation as an effective, economic and environment friendly technology for sites contaminated with petroleum hydrocarbons.

Beaudoin et al. (2000) studied the composting of hydrocarbon contaminated soil having mineral oils and grease (40% aliphatic, 32% polar, 28% aromatic 64 hydrocarbons). The results revealed that 73% of mineral oil and grease was degraded in 287 days.

Milne et al. (1998) also studied compositing with different bulking agents such as chopped barley straw, heat treated peat moss and Solv-II (a preparation of peat moss enriched with nutrients and oil-degrading microbes. Total petroleum hydrocarbons (TPHs) are reduced 25% by using barley straw and the peat moss in compositing. 55% reduction was achieved in TPHs along with high CO_2 production by the Solv-II as bulking agent. The results showed that bioaugmentation with compositing are a good remediation technique for oily residues.

Diesel oil waste mixed with biowaste (vegetable, fruit and garden waste) at a 1:10 ratio (fresh weight) degraded the biowaste mixture, four times faster than the soil alone at room temperature (Gestela et al. 2003).

Compositing of course is affected by various factors, viz. environmental conditions, microbial population present and composition of the hydrocarbon, impact of mixture composition on individual compound and overall degradation (Zytner et al. 2006).

Biodegradation experiments were conducted in sealed, 1-litre bioreactors/respirometer vessels containing soil spiked with hydrocarbon compounds in isolation and in mixtures. The influence of bacteria and fungi on the degradation process was also monitored. The degradation behavior of the various compounds was monitored using the fraction of contaminant remaining and first-order degradation coefficients based on hydrocarbon loss.

13.6 The Biodegradation of Tannery and Textile Waste

In India, approximately 3000 tanneries exist, with annual processing capacity of 0.7 million tonnes of hides and skin (Gupta et al. 2011) thus polluting 16,000 ha of agricultural land and creating drought. Tanning has been in existence for a long

time, Tannery effluents@3000 liters of waste water per 100 kg/L of processed hides produce an annual discharge of 9420 kl. These are ranked as the highest pollutants among all industrial wastes in recent years. Approximate 80% chrome tanning is used for processing leather in majority of tanning industries. Infact, only a small fraction of chromium is utilized in the process and rest is discharged in waste water directly, creating a high level of BOD, COD, electrical conductivity and heavy metals especially Chromium. Indian tanning industries generate 40 million mg/L wastes of Cr(VI) every year therefore aquatic ecosystems is the major concern over the last few decades.

Hexavalent Cr compounds, toxic metal cause health risks to humans, plants and animals due to the process of biomagnifications. Cr(VI) is a potent carcinogen to humans and animals (Matsumoto et al. 2009). High levels of Cr(VI) damages cell membranes, alter enzyme specificity; disrupts cellular functions and damages DNA structure (Bruins et al. 2000). Cr containing water used for irrigation disrupts several physiological and cytological processes in cells leading to reduced root growth, biomass, seed germination, early seedling development and induces chlorosis, photosynthetic impairment and finally leading to plant death (Chidambaram et al. 2009).

Pseudomonas sp. biodegraded and decolorized the two azo dyes, namely Congo Red (CR, Direct Red 28) and Direct Black 38 (DB 38) under anaerobic, aerobic and microaerophilic conditions which removes large amount of COD. Azo dyes after cleavage form aromatic amines and recovered amines were monitored as benzidine equivalent under anaerobic, aerobic and microaerophilic conditions (Mustafa and Delia 2003). *Effluents from tannery is generally alkaline and contaminants like BOD* (252 mg/L), COD (512 mg/L), TSS (7966 mg/L), TDS (6100 mg/L), alkalinity (454.5 mg/L), hardness (380 mg/L), chlorides (36.92 mg/L) and chromium (6 mg/L). Upon treating with them *Pseudomonas aeruginosa* there is a decrease in pH (10), BOD (47 mg/L), COD (160 mg/L), TDS (5090 mg/L), TSS (5213 mg/L), alkalinity (387.5 mg/L), hardness (195 mg/L), chlorides (27.12 mg/L) and chromium (4.8 mg/L) (Subramani and Haribalaji 2012). *Staphylococcus arlettae*, isolated from an activated sludge process in a textile industry, decolorizes four different azo dyes under microaerophilic conditions (decolorization percentage > 97%). Using a single *S. arlettae* strain in the same bioreactor, the sequential microaerophilic/aerobic stages were able to form aromatic amines by reductive break-down of the azo bond and to oxidize them into non-toxic metabolism (Elisangela et al. 2009).

13.7 Bioremediation Through Biosurfactants and Biosorption

Biosurfactants are naturally occurring in soil and they exhibit a better biocompatibility and good microbial biodegradability (Calvo et al. 2009). A wide range of surface active compounds (SACs) are synthesized by different microorganisms, which are classified according to their molecular weights, properties and localizations (Pacwa-Plociniczak et al. 2011). Role of low and high molecular weight

microbial surfactants was reported by Banat et al. (2010). Biosurfactants have low molecular weight (SACs) that lower the surface tension at the air and water interfaces that display surface activity thereby increasing the surface area of insoluble organic compounds (Abdel-Mawgoud et al. 2010). Bioemulsifiers are high molecular weight that is more effective in stabilizing oil-in-water emulsions. There by increasing the surface area available for bacterial biodegradation.

Bioremediation/biodegradation of organic pollutants rates increases by adding bioemulsifiers and biosurfactants into environment (Andrea et al. 2010). It depends on the mechanism, involved in SACs interactions between microbial cells and immiscible hydrocarbons (i) Emulsification, (ii) Micellarization, (iii) adhesion-deadhesion of microorganisms to and from hydrocarbons and (iv) desorption of contaminants.

Application of surfactants helps to increase the solubility of petroleum compounds as diesel oil biosurfactants increase oil mobility and can increase the bioavailability thereby promoting the biodegradation rates (Bordoloi and Konwar 2009). Surfactants are characterized by critical micelle concentration (CMC), hydrophilic lipophilic balance (HLB), chemical structure (Van Hamme et al. 2006). The CMC depends on surfactant structure, conductivity, or turbidity composition, temperature, ionic strength, and the presence and types of organic additives in the solutions (Fuguet et al. 2005). Micelles are capable of dissolving hydrophobic contaminants in their hydrophobic core, resulting in an increased apparent aqueous solubility of the pollutants (Prak and Pritchard 2002).

Nocardiopsis B4 actinomycetes was isolated as a potential biosurfactant producing strain using olive oil as the carbon source and phenyl alanine as the nitrogen source. The maximum production of the biosurfactant by *Nocardiopsis* occurred at a C/N ratio of 2:1 and the optimized bioprocess condition was pH 7.0, at 30 °C and 3% salt concentration. This strain plays a crucial role in the degradation of PAHs in soils. The biosurfactant activity was stable at very wide temperature, pH and salt concentrations, thus indicating its efficacy in bioremediation (Khopade et al. 2012).

13.8 Advances in Bioremediation of Complex Industrial Wastes

A challenge for industrial wastes are to degrade them through bioremediation as there is no other available technique for complete removal of these pollutants. In nature, diverse groups of microorganisms are present from long times, providing the best solution for the biodegradation and bioremediation of toxic pollutants. Different techniques such as engineering of single genes, pathway construction, alteration of the coding and controlling gene sequences, and use of recombinant microorganisms for metal removal, i.e. genetically modified biosorbents are effective for bioremediation.

Microbes have various mechanisms for removing heavy metals by adsorption to cell surfaces, complications of exopolysaccharides, intracellular accumulation, biosynthesis of metallothionins and other proteins that trap metals and transform them

to volatile compounds (Sharma et al. 2013). Genetically modified microbes and/or microbial consortia have recently been used for detoxification of environmental pollutants. Therefore bioengineering is the method other than bioremediation that helps to modify the environment (e.g., dilution, change of pH, pumped Oxygen, adding organic matter, etc.). Microbial consortia can be used in several ways e.g. by promoting growth through addition of nutrients, by adding terminal electron acceptor or by controlling moisture and temperature conditions (Smith et al. 1998). Microorganisms use the contaminants as nutrient or energy sources (Tang et al. 2007). Biofilm like mechanism in which microorganisms attached to the particles is used for non aqueous phase liquids (NAPLs) (Vallero 2010). Like other biological metal sequestering systems through genetic engineering have a higher intrinsic capability, specificity, greater resistance to environmental conditions (Majare and Bulow 2001). Conjugative plasmids, "genomic islands"(transposable elements, integrative and conjugative transposons) biodegrade the organic pollutants by the transfer of required genes.

Pseudomonas aeruginos (Valli and Suseela 2003) have ability to degrade acid blue, a diazo dye using constructed aerobic microbial consortium for decolorization and complete mineralization of azo dyes in dye laden textile effluent using photo catalytic degradation by advanced oxidation process.

Recombinant DNA technology has been used for enhanced degradation of pollutants. This technique has the potential to redesign and increase metal sequestering systems of higher intrinsic capability, specificity and greater resistance to environmental conditions (Majare and Bulow 2001). Genetic modification enhances selectivity and accumulating potential of cells (Pazirandeh et al. 1995) with production of cysteine-rich peptides, such as glutathione (GSH) (Singhal et al. 1997), phytochelatins (PCs) and metallothioneins (MTs).

These peptides bind and sequester metal ions in biologically inactive forms (Bae et al. 2001). Remediation of metal contamination is used for the over expression of these peptides in bacterial cells with enhanced metal accumulation (Pazirandeh et al. 1995).

A range of substrates have been degraded by genetically engineered microorganisms (GEMs). A study using degrading strain *Pseudomonas putida* DLL-1 was cloned by a DNA fragment including the open reading frame of *mpd* (methyl parathion hydrolase encoding gene) and cognate regulator of a methyl parathion (MP) by the shotgun method. A GEM carbofuran-degrading strain *Sphingomonas* sp. CDS-1 was successfully constructed with a broad-host vector pBBR1MCS-2 to produce a recombinant plasmid pBBR-*mpd.* CDS-pBBR-*mpd. I*t is a stable strain. It has high MP hydrolase activity (50.72 nmol min^{-1} μg^{-1} protein, which was 6.57 times higher than that of *P. putida* strain DLL-1) and has potential high for environmental bioremediation.

13.9 Reclamation and Remediation of Solid Waste

According to the third World Network reports, more than one billion pounds (450 million kilograms) of toxins are released globally in air and water. The dumping of hazardous waste like rubber, plastics, and agricultural waste into the environment is harmful to the living creatures. Solid-waste management is a major challenge in urban areas throughout the world. Continuous and uncontrolled discharge of industrial and urban wastes into the environmental sink has become an issue of major global concern (Gupta and Mohapatra 2003). The industrial and anthropogenic activities have also led to the contamination of agricultural lands resulting in the loss of biodiversity. Although the use of pesticides and herbicides increases the productivity of crop, they also increase the soil, water and air contamination (Kumari et al. 2013). Different techniques are used for solid waste remediation as:

(i) Biosparging (ii) Bioaugmentation (iii) Biopilling (iv) Land Farming

Biosparging is used for aerobic degradation and volatilization as in reducing petroleum products at underground storage tank (UST) sites. This technology was applied to a known source of gasoline contamination in order to quantify the extent of remediation achieved in terms of both mass removed and reduction in mass discharge into groundwater.

Bioaugmentation is used by addition of compound-degrading microbes or of organic amendments containing active microorganisms, e.g. activated sludge or compost. It is used for soil as well as ground water that is mainly contaminated with chlorinated ethanes, degraded into non-toxic ethylene and chloride compounds.

Biopilling is a hybrid form of composting and land farming. It includes a treatment bed, an aeration system, an irrigation/nutrient system and a leachate collection system. For proper biodegradation there should be control of moisture, heat, nutrients, oxygen and pH.

Land forming is a natural degradation process and useful mainly for pesticides. It makes a sandwich layer of excavated soil between a clean soil and that of a clay and concrete. The clean soil should be at bottom and the concrete should form the upper most layer. It is also provided with oxygen, nutrition and moisture and pH, maintained near neutral (pH 7) by using lime.

Aerobically degradable compounds (gasoline, oil and petroleum) get degraded by bioventing technique. Oxygen and nutrient like nitrogen and phosphorus is injected into the contaminated site. Oxygen is provided through low air flow rate for microbes. The rate of removal of these substances is varied according to soil texture and different composition of hydrocarbons.

Rubber is approximately 12% of constituent in the solid wastes. A rubber is non-degradable and non recyclable based on the physical composition (Conesa et al. 2004). The strength of the rubber depends on the tires that are synthetic polymers

and high grade of black carbon. It gives a large amount of toxic fumes along with carbon monoxide (Adhikari et al. 2000). Sixty five percent of decomposing rubber from vehicles, with composition, like zinc oxide that can inhibit the growth of sulfur oxidizing and other naturally occurring bacteria. This leads to slow natural degradation of rubber (Zabaniotou and Stavropoulos 2003) which requires fungus *Recinicium bicolourto* to remove the toxic component and then devulcanized by sulfur reducing or oxidizing actinobacteria like *Pyrococcus furiosus* & *Thiobacillus ferroxidans* for recycling (Keri et al. 2008). Controlled combustion of rubber is the best waste management (Conesa et al. 2004) while using the heat for energy generation.

13.10 Summary

Nature has provided various biochemical reactions and biomolecules to clean up the environment. The growing demand for sustainable technologies, such as bioremediation, help modify the environment by altering the dilution, change of pH, pumped oxygen, adding organic matter, etc. thus make it habitable. Bioremediation includes optimization by a number of biological, chemical and physical factors that help biodegrading microbes reach to conditions, balancing and controlling the movement of contaminants (microbial food). Bioremediation allows mobility and contact between the microbes and the contaminant. Two major limiting factors of any biodegradation process are toxicity to the microbial population and inherent biodegradability of the compound. Microbial methods for degradation of synthetic dyes have advantages such as high efficiency, environment friendly, and involve low operation costs. Polycyclic aromatic hydrocarbons (PAH) degradation involve the direct molecular mechanisms and genome-wide cellular ecophysiological responses. Biosufactants exhibit emulsifying, foaming, dispersing properties, detergents facilitating and desorption processes by modifying microbial cell properties/activity and have the same influence on contaminant bioavailability and the kinetics of microbial biodegradation processes. Different processes of bioremediation have been studied during the last few decades but the recent processes of genetic engineering, molecule design and nanotechnologies facilitate development of new strategies for future applications. The most common bioreactors used in bioremediation include the biofilter, the biotrickling filter, the bioscrubber and systems based on gas diffusion through suspended-growth bioreactors.

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Part III

Plant as Medicine and GE for (Plant) stress

Current Aspects of Medicinal Properties and Health Benefits of Plant *Withania somnifera*

14

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Abstract

Withania somnifera also known under the name Ashwagandha has been in use for over 5000 years. It has been concluded from studies undertaken that this plant possesses various medicinal properties contributing to several health benefits. These effects are likely to be caused due to the wide variety of steroid lactones and withanides and other components contained within the *W. somnifera* plant. Increasing number of studies has revealed the evidence for antioxidant and medicinal properties. Investigations have shown to be very positive across a wide variety of experimental systems, including in-vitro tests, cell-based studies, and in-vivo animal trials noting in particular the reduction of ROS for rats with induced Parkinson's disease and the healthy gain of weight in normal rats. There is also great interest within the anticancer abilities of *W. somnifera* and these abilities have shown to be effective against various types of cancer including prostrate and lung. Strong evidence suggests the plant also carries antibacterial activity. The aims of this article is to summarize current research dealing with medicinal properties and health benefits of *Withania somnifera* extracts with a focus on antioxidant, anticancer and antimicrobial properties.

Keywords

Withania somnifera · Ashwagandha · Antioxidant · Antibacterial · Anticancer

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14.1 Introduction

Withania somnifera (shown in Fig. 14.1) is a medicinal plant, which belongs to a group, called the Solanaceae family of plants and is known under the names Indian ginseng or Ashwagandha. The Solanaceae plant family is constructed of over 2700 plant species, which are divided into 98 separate genera. Authors of this chapter started working on this plant few years back, some of the results have been published on its medicinal properties (Barnes et al. 2015). This chapter has included information collected from important research conducted with general health effects of *Withania somnifera* extracts, antioxidant properties, anticancer and antioxidant activity.

A search of Medline/PubMed and ISI web of science databases showed the following results; full articles for *Withania somnifera* (940), reviews (54), controlled clinical trials (16) – of which 13 were randomized clinical trials. Currently there appeared to be five (5) systematic review and meta-analysis on the topic. In the following narrative review, the aim is to summarize current research dealing with medicinal properties and health benefits of *Withania somnifera* extracts with a focus on antioxidant, anticancer and antimicrobial properties. Previous reviews dealing with the pharmacological and health aspects of *Withania somnifera* include those by the following authors (Chen et al. 2011; Kulkarni and Dhir 2008; Mishra et al. 2000; Sharma 2013; Singh et al. 2010; VandenBerghe et al. 2012; Winters 2006).

Fig. 14.1 Picture of the plant *Withania somnifera.* (Source: http://xyerectus.com/wp-content/uploads/2013/11/390651_090421122758_Withania_somnifera4.jpg)

14.2 Health Benefits

Records showing the medicinal use of *W. somnifera* date back over 5000 years and are related to a traditional Hindu methodology of medicine, known as Ayurvedic medicine. In systems such as Ayurvedic and Unani medicine, extracts from plants such as *W. somnifera* claim to have multiple health benefits in the treatment of a variety of health problems. Observational and ethnographic evidence suggests that *Withania somnifera* has been used for the treatment a range of ailments: muscle tension, bone damage, aging and dehydration amid several others. Observations also suggest that the plant contains additional pharmacological properties, which can be useful in modern medicine, such as tackling neurodegenerative disorders (e.g. Alzheimer's disease) and addressing declining cognitive function in geriatric patients, amid a plethora of other ailments.

Interests in the health benefits of *Withania somnifera* has accelerated recently with growing numbers of discussions focusing on, Parkinson's and neurodegenerative diseases (Durg et al. 2015; Srivastav et al. 2017; Yenisetti et al. 2016), cancer and neoplasms (Lee and Choi 2016; Palliyaguru et al. 2016; Rai et al. 2015) as well as, other generic chronic diseases (White et al. 2016). Moreover the number of carefully desgined controlled, human trials are now groiwng but more work can be anticapated in future years (Table 14.1). It is suggested that the reason for a majority of the associated health benefits is due to the volume and variety of chemical compounds, which can be found within specimens of *WS* (Table 14.2).

Table 14.1 A summary of some human trials using *Withania somnifera*

Condition, outcome	Study	+RE
Osteoarthritis pain	Kulkarni et al. (1991)	+
Hypoglycaemic, cholesterol lowering	Andallu and Radhika (2000)	+
Sleep quality	Manjunath and Telles (2005)	0
Depression	Krishnamurthy and Telles (2007)	0
Anxiety	Cooley et al. (2009)	+
Immunologic effects	Mikolai et al. (2009)	+
Balance & cerebellar ataxias	Sriranjini et al. (2009)	+
Reproductive health	Ahmad et al. (2010)	+
Innate Immunity	Bhat et al. (2010)	+
ADHD	Katz et al. (2010)	+
Schizophrenia	Agnihotri et al. (2013)	+
Chemotherapy associated fatigue	Biswal et al. (2013)	+
Bipolar disorder	Chengappa et al. (2013)	+
Male reproductive health:	Gupta et al. (2013)	+
Bone mineralization	Mirakzehi et al. (2013)	+
Anxiety, Stress	Pratte et al. (2014)	+
Women reproductive health	Dongre et al. (2015)	+
Muscle strength	Wankhede et al. (2015)	+
Obsessive compulsive disorder	Jahanbakhsh et al. (2016)	+
Body weight management	Choudhary et al. (2017)	+

+Promising or positive results, (0) No result, (−) Negative results

14.3 Components in Withania somnifera

The phytochemical constituents of *WS* root extract were classed into 6–8 chemical classes (Table 14.2); alkaloids, steroidal lactones, steroids and phytosterols, salts, flavonoids, and nitrogen containing compounds. Results from phytochemical screening studies showed *WS* root to contain, some eight classes of compounds depending on the solvent used for extraction; alkaloids, flavonoids, steroids/ phytosterols, terpenoids, saponins, tannings, phenols and various glycosides (Visweswari et al. 2013). Overall, 12 different alkaloids and 30+ withanolides isolated from *WS* (Singh et al. 2010). Currently, the steroidal-lactones have received most attention. The most common withanolides e.g. Withanone, Withaferin A, Withanolides are the basis for some of the various health benefits associated with the plant. Withaferin A has been considered as one of the more important compounds as proof has shown it

Table 14.2 Pharmacological components from *Withania somnifera*

Classes	Examples	Clinical effect	Biological activity
Alkaloids	Withanine, Withananine, Withasomnine, Somniferine, Tropeltigloate, Somniferinine, Somninine Nicotine	Anti-Microbial, Sedative, Anti-Spasmodic; Anti-Cancer, Leg Cramps, Anti-Diarrhoea, Psychiatric, Palpitation	Anti-inflammatory Antioxidant Anti-inflammatory Apoptosis inducing Diuretic Sedative/anxiolytic Immunomodulatory
Steroidal lactones	Withaferin-A, Withanone, Withanolides	Anticancer	Anti-bacterial Anti-Stress Cardio-protective Neuroprotective Anti-hypertensive Anti-arthritic Anti-cancer Anti-diabetic
Steroids, phytosterols	Cholesterol, B-Sitosterol, Stigmasterol, Diosgenin, Stigmastadien, Sitoinosides VII, Sitoinosides VIII, Sitoinosides IX, Sitoinosides X	Aphrodisiac, Lowers cholesterol, Immune anti-tumour	
Salts	Cuscohygrine, Anahygrine, Tropine, Pseudotropine, Anaferine	?	
Flavonoids	Kaempferol, Quercetin	Antioxidant, CVD risk dec. osteoporosis, cholesterol lowering cancer risk dec.	
N-Compounds	Withanol, Somnisol, Somnitol		

Adapted from Dar et al. (2015), Visweswari et al. (2013), and White et al. (2016)

to be therapeutically active (Mirjalili et al. 2009; Misico et al. 2011; Sangwan et al. 2014; White et al. 2016; Zhang et al. 2014).

The alkaloids from *WS* remain quite illusive, as no structural formulas appear to be available. Nevertheless, some classical pharmacognosy sources suggest that *WS* alkaloids crystalize from chloroform extracts and that the majority (38%) consist of withanine ($C_{44}.H_{80.}\ O_{12}.N_{2,}$ predicted FW = 828) which has a distinctive sedative and hypnotic activity (Atal 1975; Glasby 2012). More recently, WS alkaloids were prepared quantitatively using a mixture of acetic acid and methanol (Takshak and Agrawal 2014).

14.4 Antioxidant Activity of WS Root Extract

Currently about 114% of the literature addressing WS is associated with antioxidant activity in one way or another, the majority of papers were reviews, or assessed antioxidant activity in-vitrousing small rodents (Devkar et al. 2014; Dhanani et al. 2013; Fernando et al. 2013). A great interest lies within testing any antioxidant activities that *W. somnifera* extracts may possess, as they can aid reduction of oxidative damage to cells, effectively acting in order to decrease the likelihood of degenerative diseases such as the development of Alzheimer's disease. Increased damage levels due to oxidation to cell types can be partly responsible for a spectrum of chronic diseases liked with increased oxidative stress, e.g. Alzheimer's. This damage takes place either by hydroxyl radicals causing damage to mitochondrial DNA, or by protein oxidation into carbonyls (Scartezzini and Speroni 2000). In this section, we review briefly evidence for antioxidant content for *WS* with a focus on roots. For a lack of space, only primary studies after 2005 are considered.

14.4.1 *In-Vitro* Tests

Several simple chemical tests and enzymatic tests were performed to determine the content of non-enzymatic antioxidants and enzymatic antioxidants for *WS*. With respect to chemical tests in use the most common of these that are used in testing antioxidant samples include, β-carotene bleaching test (BCBT), DPPH, ABTS, FRAP and Folin method of testing total phenols. These tests are deemed state of the art due to their effectiveness, simplicity and repeatability meaning that they are in the most usage. They have maintained their usefulness over time because of their success rate in the analysis of different areas of antioxidant activity including primary and secondary antioxidant activities as well as the ability of free radical scavenging. These various In-vitro tests also remain useful as they can test a variety of antioxidant activity properties, this is important as no individual antioxidant test known will cover all the aspects and descriptions of activity (Aruoma 2003; Fraga et al. 2014; Frankel and Meyer 2000; Koleva 2002).

Non-enzymatic antioxidants associated with *WS* include vitamin C, vitamin E, glutathione, carotenoids, lycopene, total phenols and flavonoids (Jaleel 2009). The

current research showed that *WS* also contained significant activity for several enzymatic antioxidants, including catalase, superoxide dismutase, and various peroxidases (Jaleel 2009; Kanungo et al. 2013; Sumathi and Padma 2008). Further studies involved so-called total antioxidant capacity measured in terms of radical quenching assays (DPPH, ATBS method), total phenols, iron (III) reduction or FRAP methods (Alam et al. 2012; Dhanani et al. 2013; Fernando et al. 2013). The investigations performed using solvent extracts, showed that *WS* contains high antioxidant capacity, though values depend on the solvent choice for extract, and whether analysis is performed using different botanical parts of the plant (leave, berries, and roots etc.).

14.4.2 Animal Trials Testing for *W. somnifera* Antioxidant Ability

In vivo animal trials have been used for additional observations on the antioxidant effects of *W. somnifera* extracts, usually leading to protection from drug-induced reactive oxidative stress (ROS) or other insults. Investigations using rats as models showed WS antioxidant activity and protection from a variety or ROS:adjuvant induced arthritic ROS (Rasool and Varalakshmi 2007), doxorubicin (Hamza et al. 2008), gentamicin ROS (Jeyanthi and Subramanian 2010), swimming induced ROS (Misra et al. 2009), alloxan toxicity (Udayakumar et al. 2010), carbon tetrachloride ROS (Elberry et al. 2010), radiation induced ROS (Hosny et al. 2012), bromobenzene nephrotoxicity (Vedi et al. 2014).Such trials carried out on both rats and mice are a common methodology associated with the extract and various conditions have been shown to be successfully treated with the use of the plant extract. It would seem that specific compound from WS possess antioxidant capacity such as flavonoids and phenols (Keshavkant et al. 2008) and other less well defined components demonstrated using TLC-DPPH method (Devkar et al. 2014).

Gupta and co-workers found that *WS* extract prevents Cu induced oxidative stress in the brain of rats (Gupta et al. 2003). Mohanty and co-workers concluded that WS produces a cardio-protective effect in rats due to the activation of endogenous antioxidants (Mohanty et al. 2004). WS extracts could reduce the severity of stress-induced gastric ulcer in rat by an antioxidant mechanism (Bhatnagar et al. 2005). Additional animal-based evidence generally support the consensus that *WS* administration is protective effects with regards to stress and/ or drug induced oxidative stress (Al-Qirim et al. 2008; Jeyanthi and Subramanian 2010; Jeyanthi et al. 2010).

In a rat-experimental model for Parkinson's disease, intra-cranial injections with 6-hydroxydopamine (6-OHDA) were used to produce chronic neurotoxicity by increasing ROS. Pre-treating rats with *WS* prior to 6-OHDA prevented neurobehavioral decline, as well as reducing biological markers for oxidative stress in a dose-dependent manner (Ahmad et al. 2005).*W. somnifera* treatment also reduces oxidative damage caused in rats induced with type II diabetes, the extract has proven to increase anti-oxidative enzyme activity such as GPX and CAT amongst various others. This carries statistically significant results when delivery happens in high doses of over 200–400 mg/kg. As well as involvement in level decreases of lipid

peroxide, these studies were proven with the use of well-established tests including NADPH oxidation and the analysis of lipid hydroperoxide levels by testing with thiobarbituaric acid, a complex was then formed due to the lipids involved becoming peroxidised (Anwer et al. 2012).

Recent findings that WS compounds activate nuclear factor erythroid 2-related factor 2 (Nfr2) pathway (Heyninck et al. 2016; Sun et al. 2016; White et al. 2016) may help explain some of the antioxidant and detoxification results observed with animal studies. Briefly, Nrf2 functions as cellular sensor for mild ROS arising from the metabolism of antioxidants by cells. Following its activation by oxidizable polyphenols, quinones, lactones, ROS or electrophilic agents, thenNrf2 is transferred from the cytoplasm to the cell nucleus. Arriving in the cell nucleus Nrf2 binds to a so-called antioxidant responsive element (ARE) thereby increases the expression of genes for phase I and phase II drug metabolism enzymes, antioxidant enzymes (CAT, SOD, GSH-Px), drug detoxification transporters (Niture et al. 2014). Activation of Nrf2 is invoked to explain the protective role dietary phytochemicals for many conditions liked with ROS and chronic inflammation, e.g., arthritis, cancer, diabetes, neurodegenerative disease, (Qin and Hou 2016). NrF2 activation is also associated with the suppression of an inflammatory responses believed to be controlled by another nuclear factor kappa-beta (NFKB) including the synthesis of inflammatory cytokines (Ahmed et al. 2017). It should be noted that NrF2 activation can lead to increased resistance not only to toxic chemicals but also some therapeutic cancer drugs (Kensler and Wakabayashi 2010). It can be anticipated that more research focusing WS and Nrf2 system will be forthcoming.

14.5 Anticancer Activity of WS Root Extract

There is rapidly increasing interest in the creation and use of new anticancer treatments. With cancer accounting for over 25% of deaths in humans annually, treatment using natural biomaterial such as *W. somnifera* are being investigated due to the partial ineffectiveness and side effects of a negative nature that are associated with modern day treatments for cancer including chemo and radiotherapy. The anticancer properties of WS and individual chemical components were reviewed recently (Winters 2006; Vyas and Singh 2014; Lee and Choi 2016; Palliyaguru et al. 2016; Rai 2015). In the following section, we consider recent investigations focussing on WS whole extract rather than single components. Recent investigation using WS root extract found anticancer activity against many human cell lines including, liverHep2 cells (Mathur et al. 2006), colon HCT15 cells, prostateDU-145, PC3 cells, lung A-549 cells and neuroblastoma IMR-32 cells (Yadav et al. 2010);skin melanoma cells (Halder et al. 2015), breast MDA-MB-231 and MCF-7 cells (Khan et al. 2015; Khazal et al. 2013; Maliyakkal et al. 2013). In general terms, the cytotoxicity of WS extract (25–50 ug/ml) was significant and was affected by the type of solvent used for extraction, cell type and by the length of the exposure time (24–72 h). Studies consistently showed the role of WS extract in disrupting cell-cycle progression.

14.6 Antibacterial Activity of WS Root Extract

Due to rapidly increasing and widespread antibiotic resistance, there is an increased global demand for new and innovative treatments. These treatments should be able to tackle diseases and infections that some antibiotics no longer can due to resistance. There is a continuing demand for fresh antibacterial treatments to be developed as incidence rates are on the rise around the world; this demand has led to the resurgence of *WS* use and testing in order to develop new ways of fighting against several diseases. The antibacterial effects of *W. somnifera* are continuously being tested with studies dating back to the 1950s (Kurup 1958).

However, investigations that are more recent have shown prominent outcomes and a better understanding of the antibacterial mechanisms present. In this section, we consider current research related to the antibacterial properties of WS extracts prepared from whole root powder and not individual components, though research shows specific compounds to be active (Girish et al. 2006; Shanazbanu et al. 2006). A search using PubMed, and ISI web of science databases found 45 and 57 references (respectively) that describe Withania S (title) antibacterial or antimicrobial properties (topic). The lists of papers were hand sorted, and duplicates removed. Publications were supplemented also with papers from Google Scholar and a total of 10–11 papers were recovered that described antibacterial activity of WS extracts prepared from roots (Table 14.3). There were no previous reviews focussing exclusively on the antibacterial properties of *W. somnifera*.

As noted above (Table 14.3), a variety of studies have been carried out dealing with antibacterial activity with the use of various different methodologies, with some studies using extracts from both the root and leaves of *W. somnifera*. In general, the approaches adopted by different investigators involve preparing dried materials, size reduction by gridding, and extraction of biologically active components using water or a low molecular weight non-aqueous solvent. The extracts are concentrated using a rotary evaporator or air-drying, and then investigated for antibacterial activity using a spectrum of bacterial specimens.

Another recent study uses the methanolic, aqueous, chloroform, acetone and ethanolic extracts in a disk diffusion methodology to test human pathogenic bacteria, including *MRSA, E. faecalis, S. pyogenes, K. pneumoniae* (Rizwana et al. 2012). The study concludes that extracts from *W. somnifera* including those from the root, stem and leaves show a great potency against different bacterial species. Overall looking at the information within the study antibacterial levels are high especially from extracts taken from the leaf of the plant, followed by the stem and then lastly the root, especially in alcohol and acetone based extracts. Taking into account that aqueous extracts also produced notable effects; polar solvents however proved to produce the best overall antibacterial effects and present the opportunity for future options in this area (Rizwana et al. 2012).

Other studies also give a contribution to these results as well as adding relevant information, one such study suggests how inhibition zones change in size based on a dose dependent manner notably from leaf based extracts. A direct comparison to an established antibiotic- chloramphenicol, where extracts are shown to be

Table 14.3 Antimicrobial and antibacterial activity of *Withania somnifera* extracts

Part	Solvent	Microorganisms	Method	References
Leaves, Root	Methanol, Hexane, Diethyl Ether	*Salmonella typhimurium, Escherichia coli.*	Disc diffusion	Arora et al. (2004)
Root, Leaves	Hexane, Ethyl Acetate Methanol Water	*E. coli, S. aureus S. typhimurium*	Agar well diffusion, Mice	Owais et al. (2005)
Root	Benzene Ethanol	*S. typhi, S. aureus, S. rubidaea, Lactobacillus* sp.		Dar et al. (2008)
Bark, Leaves, Root	Methanol	*B. subtilis, E. coli, P. fluorescens, S. aureus X. axonopodis* pv. *A. flavus, D. turcica, F. verticillioides*	Disc diffusion	Mahesh and Satish (2008)
Root	Water, Methanol	*S. aureus (MDR), 15 local strains, 5 patient strains*	Agar well diffusion	Datta et al. (2011)
Root	Water	*S. aureus* (methicillin resistant)	Agar well diffusion	Mehrotra et al. (2011)
Whole Plant	Ethyl Acetate	*S. aureus, E. coli, P. aeruginosa, B. subtilis*	Agar well diffusion	Sundaram et al. (2011)
Fruit Leaves, Root	Water + 80%, Methanol	*E. coli, S. typhi, C. freundii, P. aeruginosa, K. pneumoniae,*	Agar well diffusion	Alam et al. (2012)
Leaves, Roots, Stem	Acetone, Chloroform Ethanol, Methanol	*B. subtilis, E. coli, E. feacalis, MRSA, S. pyogenes, P. aeruginosa K. pneumoniae*	Agar well diffusion	Rizwana et al. (2012)
Roots	Methanol	*Streptococcus mutans, S. sobrinus S. oralis, A. naeslundii*	Absorbance 550 nm	Pandit et al. (2013)
Root	Water	*E. coli*	Disc diffusion	Kumari and Gupta (2015)

Investigations using only leaf extracts were excluded

increasingly potent by around a factor of 5 with a lowered level of toxicity, in this case where 4 µg of an extract does the same as 20 µg of an antibiotic. There is some focus from this study given to prophylactic effects on animals, rats induced with the infection *S. typhimurium* were given 100 mg/kg doses of *W. somnifera* as a form of treatment. This as a method proved to be successfully effective and leads to the belief that future prospects for *W. somnifera* are good and in future there may be a way to find a niche allowing the plant to be capable of treating infections caused by

bacteria in humans, however this can only happen if effective human clinical trials can be carried out (Owais et al. 2005).

These studies concluded that the *W. somnifera* was very potent and proved highly effective against strains including *E.coli, B. subtilis* and *P. aeruginosa*, where during tests 14–16 mm inhibition zones were formed. Most investigations used agar well diffusion assays or the disc diffusion assays as this way of testing is considered gold standard, fast and easy (Hombach et al. 2013) Modern systems that can be used for effective screening of the susceptibility of bacteria include the BACTEC system which uses fluorescent markers along with CO_2 to measure bacterial sensitivity (Collins and Franzblau 1997). These systems also included a recent development where 96-well microplates are used in the determination of bacterial susceptibility and MICs with the capability of using small concentrations of plant material such as *W. somnifera* extracts as low in volume as 25 µl (Eloff 1998).

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Active Compounds and Bacteria Harbouring Capacity of Lichens and Its Medicinal Use in Bacterial and Cancer Infections

15

Narendra Kumar and S. M. Paul Khurana

Abstract

Lichens are composite organisms formed upon symbiotic association of fungi and algae doing photosynthesis (blue green algae). Lichenized fungi produce unique secondary metabolites which bears a wide spectrum in biological activities viz., anti-HIV, production of antibiotics, anti-protozoan, anticancer etc. This review focuses primarily on the antibacterial activity of lichen's secondary chemicals related to major antibacterial activity and highlights of each study. The literature published clearly demonstrates that the lichen extracts and their constituent compounds have potential of significant inhibitory activity against various pathogenic bacteria viz., *Bacillus cereus, Bacillus subtilis, Chrysobacterium indolthecium, Clavibacter michiganensis* subsp. Michiganensis, *Enterococcus faecalis, Erwinia carotovora* sub sp. carotovora, *Escherichia coli*, Methicillin-resistant *S. aureus*, *Pseudomonas aeruginosa. Pseudomonas cichorii, Pseudomonas savastoni* pv. Fraxinus, *Pseudomonas syringae* pv. syringae, *Pseudomonas vesicularis, Staphylococcus aureus, Staphylococcus aureus, Streptococcus pneumoniae, Xanthomonas axanopodis* pv. Malvecearum, *Xanthomonas hortanum* pv. pelargonii, *Xanthomonas phaseoli, Yersiniapseudotuberculosis,* even at low concentrations. The literature records however shows no studies reported anything on the specific mechanism of action against pathogenic bacteria. Lichens harbours many biologically active compounds in which less numbers have been experimentally tested. It needs very deep studies for search of active compounds. It needs to do more clinical trials and search of mechanism of action for potent compounds in lichens.

Keywords

Antibacterial activity · Secondary metabolites · Lichens · Biological activity

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15.1 Introduction

Pharmacological molecules are the raw material for Pharmaceutical industries and for increasing new infections the industries may be forced for development of a new molecules (Santiago et al. 2010). For this Lichens can serve as an ideal source of bioactive metabolites. Lichens form symbiotic associations through green algae, fungi and even through cyanobacteria. They have wide variation in their chemistry. They can result in production of many polyketide derived components for example depsides and depsidones which is not found elsewhere. It have been well appreciated in traditional medicinal practices. But due to their ignorance in modern pharmaceutical industry that may be due to difficulties for developing axenic cultures it got less attention.

Lichens are non-vascular cryptogams, comprising a self-supporting, symbiotic association between a photobiont (an alga or a cyanobacterium) and a mycobiont (an ascomycetes or basidiomycetes fungus). They have the most stable and successful symbiotic interactions. Lichens are cosmopolitan and found in almost every type of habitat on earth from a Arctic region to deserts, high elevations/mountains, tropical or temperate forests etc. Together with mosses, lichens form a dominant group of organisms covering over 8–10% of terrestrial habitats, especially higher elevations. They are capable of growing on various substrates viz., rock (saxicolous), bark (corticolous), soil (terricolous), plastic (plasticolous) and leaves (follicolous). Lichens occur in any one of the three morphological forms such as crustose (spreading on upper surface of substratum), foliose (leafy and often loosely bonded to substratum) and fruticose (bush like hanging attached to substratum at a single point).

They occur poorly in the industrial zones or big cities (Heganuer 1962). Lichens play a major role in food and drug industries (Richardson 1988). Lichens are considered as one of the best indicators of air quality. Since time immemorial, lichens have been used as sources of food, spice, medicine, and dye. Lichens have been a part of traditional medicine and used to treat several human and veterinary ailments by various tribes of several countries (González-Tejero et al. 1995; Devkota et al. 2017).

It is now well known that penicillin have fungal origin. Lichens comes in a category of organisms which have wide medical applications (Crockett et al. 2003). There was a lot of interest for antibiotics during the World War II upto 1950s. Scientists are paying special attention for Antibiotic properties in lichens. A study revealed that 50% of all lichens show antibiotic properties (Malhotra et al. 2007). Burkholder established that lichens are a good source for antibacterial activity. He evaluated 42 lichens for their antibiotic in which 27 showed growth inhibitory activity against bacteria (Burkholder 1944).

The unique and biologically active fractions present in the lichen thalli contribute to several important medicinal values of lichens, like antiviral, antibacterial, antifungal, antioxidant, cytotoxic effect and many more. The manifold properties of lichen metabolites represent their therapeutic potential having a great impact on the industries (Ramya and Thirunalasundari 2017).

The development of resistance in bacteria is now one of the global problems. Bacteria develop resistance to antibiotics in a relatively shorter period of time making them very difficult to control. Bacteria such as *Staphylococcus aureus, Streptococcus pneumoniae, Enterococci, Pseudomonas aeruginosa, Klebsiella pneumonia, Mycobacterium tuberculosis* and *Escherichia coli* are among the many drug resistant bacteria. The nonjudicious use of antibiotics and the ability of pathogenic bacteria to spread the resistance gene to the susceptible strains by genetic means are resulting in gradual increase in resistance. Besides, most of the antibiotics are costly and their much use is often associated with certain adverse health effects. Nowadays, the scientific community is focusing more on the search for alternatives for disease therapy. Natural products such as plants, microbes and lichens have been investigated and are found to be promising alternatives (Hoskeri et al. 2010; Agboke et al. 2011; Vivek et al. 2014).

Lichens are slowest developing symbiotic formations having algal and fungal associations. The slow developing organisms occupy least habitats, harbours higher levels of defense chemicals for defending themselves against various attacks. The lichens results in production of more than a 1000 different secondary metabolites (Coley 1988). Which shows many useful attributes viz., protection for a wide spectrum of microbes, animal predators, creating defense for environmental stress like desiccation and UV radiation, in physiological control of metabolism. These are useful to increase permeability for algal membranes and with a up in permeability for flow of nutrients in fungal parts. The lichens have potential to protect themselves from attack of microbes viz, bacteria, nonlichenized fungi, nematodes. So for use of lichens metabolites may be used for general research having good future.

It produces two types of metabolites primary and secondary. Primary shows structural functions for their roles in cellular metabolism. These are intracellular in origin synthesized independently. And consists of lichenin, hemicelluloses, chitin (in hyphal walls), isolichenin, pectins, polyalcohols, disac-charides, enzymes, amino acids and pigments viz., chlorophyll, b-carotenes, xanthophylls, etc. (Podterob 2008). The fungal partner produces secondary metabolites which are exported outside of hyphae. This was deposited in the form of crystals in various parts of the thallus mainly in the upper cortex in specialized structures in the form of fruiting bodies.

The aim of this chapter is to collect information regarding active chemicals present in Lichens and to collect details regarding the antibacterial investigations of lichens against pathogenic bacteria.

15.2 Diversity

This is the fungal component which creates basis for classification of lichens. On the basis of fungal species their binomial names are given. The alga possess their scientific name which have no relation with the lichen or fungus. The total lichen species are 13,500–17,000 which has been studied and identified. India have a diversity of 2368 species. This presents 18% of world total lichen population (Rai

et al. 2014). In which 20% of known fungal species are have relation with lichens. Their largest number are from the Ascomycota containing 40% of species which is in such association. Some of comes in orders of nonlichenized fungi and live in form of saprotrophs or parasites on plants (for example the Dothideales, Leotiales and Pezizales). Other lichen fungi comes in five orders which show symbiosis (Orders Gyalectales, Graphidales, Teloschistales, Peltigerales, Pertusariales). Finally we can say 98% of lichens show as comycetous mycobiont. The other next category of lichenized fungi belongs to unassigned fungi imperfecti whose sexual form of reproduction are not well known. Inspite of this a few Basidiomycetes are lichenized which include agarics for example *Lichenomphalia*, clavarioid fungi (*Multiclavula*) and corticioid fungi (*Dictyonema*) (Kirk et al. 2008).

On the basis of formation of colony lichens may be crustose, foliose and fruticose. The Crustose lichens forms a crust which strongly adheres on the substrate (bark, soil, rock.). This makes separation impossible from the substrate without destruction (Fig. 15.1a, b). Foliose lichen have flattened leafy thalli with upper and lower cortex. This adds numerous layers which are stratified (Fig. 15.1c). A **fruticose have** a coral like shrubby or bushy growth structure made up of a thallus and a holdfast (Fig. 15.1d).

Fig. 15.1 (**a**) Colony of Crustose lichens on a wall; (**b**) crustose lichen on a tree trunk; (**c**) Foliose lichen -*Flavoparmelia caperata* on a branch of a tree; (**d**) Fruticose lichen -*Letharia vulpina*

15.3 Studies on Diversity of Bioactive Compounds in Lichens

A perusal of Literature revealed that most lichen substances are dibenzofuranes, phenolic compounds (orcinol and b-orcinol derivatives), lactones (e.g. protolichesterinic acid, nephrosterinic acid), depsides (e.g. barbatic acid) depsidones (e.g. salazinic acid), usnic acids (e.g usnic acid), depsones (e.g. picrolichenic acid), quinones (e.g. parietin) and pulvinic acid derivatives (e.g. vulpinic acid). This have many uncommon compounds viz., a cyclic depsipeptide -arthogalin (Huneck and Himmelreich 1995), cytotoxic scabrosin esters obtained from *Xanthoparmelia scabrosa* (Ernst-Russell et al. 1999), brominated depsidones (Rezanka and Gushina 1999), brominated acetylenic fatty acids (Rezanka and Dembitsky 1999) and monotetrahydrofuranic acetogenin derivatives (Rezanka et al. 2004), g-lactonic aliphatic acid glycosides (Rezanka and Gushina 2000, 2001a, b), xanthone glucosides (Rezanka et al. 2003) and mycosporine collemin A extracted from *Collema cristatum* (Torres et al. 2004).

Pavlovic et al. (2013) isolated four main lichen acids from *Hypogymnia physodes* viz., physodalic acid, physodic acid, 3-hydroxy physodic acid and isophysodic acid. Kosanić et al. (2014) extracted two main compounds such as atranorin and fumarprotocetraric acid from acetone extracts of *Cladonia pyxidata, Cladonia furcata* and *Cladonia rangiferina*. Fernandez-Moriano et al. (2015) found fumarprotocetraric acid, usnic acid, pinastric acid and vulpinic acid in methanol extracts of two Parmeliaceae lichens: *Cetraria islandica* and *Vulpicida canadensis* using HPLC. Sahin et al. (2015) found evernic, fumarprotocetraric, lecanoric, stictic and usnic acids in Ramalina viz., *R. farinacea, R. fastigiata* and *R. fraxinea*. Honda et al. (2016) reported that *Parmotrema screminiae* acetone extract contains norlobaridone, protolichesterinic acid, and atranorin acid. The chemical Benzoic acid, 2, 4 dihydroxy, 6 methyl-methyl ester was extracted in *Rocella montagnei* (Shanmugam et al. 2016). While Delebassée et al. (2017) found the cytochalasin E in *Pleurosticta acetabulum.*

Thadhani and Veranja (2017) reported ubiquitous compounds viz., zeorin, methylorsellinate, methyl-β-orcinol carboxylate, methyl haematommate, lecanoric, salazinic acid, sekikaic acid, usnic acid, gyrophoric acid, and lobaric acid while vulpinic and usnic acid and pinastric acid was reported by Béatrice et al. (2017). While Musharraf et al. (2017) mentioned that approximately 1050 secondary lichen compounds have been identified.

15.4 Lichens, a Promising Source of Medicines

For centuries, many folk medicines from different countries are useful in various ailments (Dayan and Romagni 2001). It is well written in Ayurvedic and Unani texts to treat ailments such as leprosy, stomach disorders, asthma inflammations, bronchitis, blood and heart diseases, scabies etc. (Shukla et al. 2010). They show many Medicinal features such as antibiotics (Paudel et al. 2010), antioxidants (Gulluce et al. 2006; Bhattarai et al. 2008) antiHIV (Nakanishi et al. 1998), anti-cancer (Ren

et al. 2009), antiproliferative (Bucar et al. 2004; Burlando et al. 2009) and antiprotozoans (Schmeda-Hirschmann et al. 2008).

15.4.1 Antibacterial

Burkholder et al. (1944) did an experiment for the antibiotic activity in lichens. They found efficacy of 42 lichens useful against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli.* 27 lichens were found active against *S. aureus, B. subtilis.* But it was ineffective against *E. coli.*

Lichen species are active against many gram-positive pathogenic bacteria (Table 15.1). Compounds such as Ñvulpinic acid, lichesterinic acid, usnic acid, pulvinic acid derivatives and orcinol type depsides and depsidones show valuable antibiotic actions. These compounds are effective against many multidrug resistant bacteria (Kokobun et al. 2007; Martins et al. 2010). It can enhance formation of bacterial biofilms (Francolini et al. 2004). It also have synergistic effects (Safak et al. 2009). The inhibitory concentration of lichens are lower in comparison to any other sources of antibiotics till date (Weckesser et al. 2007; Gordien et al. 2010).

In Lichens around 1050 secondary metabolites have been well known But antibacterial testing has been done only in 50 species. Vartia (1973) mentioned that more than 50% of the lichens have antibiotic potential. It needs in depth efforts for screening the lichen extracts in general along with screening of specific lichen metabolites.

15.4.2 Anticancer Activity

The potential of a purified lichen extracts was evaluated against Albino wistar rats which was cancer induced under in vivo condition. The Lichen *Parmotrema reticulatum* showed highest control in cervical cancer cell line model. This Cancer cell line model showed reduction in tumor proliferation when it was treated through bioactive anticancer lichen compounds and compared with a standard drug (Shanmugam et al. 2016).

Secondary metabolites of lichens contains cycloaliphatic, aliphatic, terpenic and aromatic compounds which is unique for higher plants. Because of the presence of these compounds these have interesting pharmacological and biological actions. But only a few have been tested for their potential against many in vitro cancer models.

The cytotoxicity of physodic acid, atranorin and gyrophoric acid metabolited extracted from Lichens have been tested against A375 melanoma cancer cell line. The depsides atranorin and gyrophoric acid resulted lower activity against melanoma cancer cells. While depsidone physodic acid produced dose response activity at the range 6.25–50 μM concentrations in A375 cells. This activated apoptotic process which involved reduction in Hsp70 expression (Cardile et al. 2017). But Delebassée et al. (2017)recorded cytochalasin E of *Pleurosticta acetabulum* a potent inhibitor of human HT29 colorectal cancer cells.

Table 15.1 Present scenario of antibacterial activity of Lichens

Name of Lichens/metabolites	Tested against Bacteria	Results	Reference
Physcia americana, Parmotrema perlatum, Hypogymnia phylodes, Lepraria ecorticata	Oral microorganisms (gram positive bacilli) of both herbivorous and carnivorous animals such as dog, cat, cow, hen and rabbit	Strong antimicrobial activity, the zone of inhibition found more in the species of *Parmotrema perlatum* from southern zone of Karnataka, Coorg	Priyadarshini et al. (2017)
Himalayan lichen viz. *Bulbothrix setschwanensis* (Zahlbr.) extracted in acetone, chloroform and methanol	Six clinical bacterial strains (*Enterococcus faecalis, Escherichia coli, Listeria monocytogens, Staphylococcus aureus, Streptococcus pyogens, Vibrio cholera*)	Acetone extract showed promising antimicrobial activity against *S. aureus* (1.56 mg/ml) and *Cryptococcus neoformans* (6.25 mg/ml).	Maurya et al. (2017)
Ethyl acetate extracts of *Cladonia stellaris, C. pyxidata* and *C. furcat*	*Pseudomonas vesicularis, Clavibacter michiganensis* subsp. michiganensis, *Pseudomonas cichorii, P. savastoni* pv. fraxinus, *Xanthomonas axanopodis* pv. malvecearum, *Chrysobacterium indolthecium*, *Pseudomonas syringae* pv. syringae, *Erwinia carotovora* subsp. carotovora, *Xanthomonas phaseoli* and *Xanthomonas hortanum* pv. pelargonii isolates	Variable degrees of antibacterial activities of with inhibition zones of to 6–25 mm.; maximum antibacterial activity was found in *C. stellaris* extract (25 mm) stronger than the positive controls (ampicillin and tetracycline) against *P. syringae* pv. syringae and *Erwinia carotovora* subsp. carotovora.	Sokmen et al. (2017)
Thirty-two extracts from eight lichen species viz., *Heterodermia leucomelos, Cladonia subradiata*, *Parmotrema tinctorum*, *Leptogium* sp., *Parmotrema crinitum*, *Herpothallon* sp., *Parmotrema reticulatum* and *Ramalina celastri*	Bacterial strains *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris* and *Staphylococcus aureus*	Methanolic extracts had the maximum efficacy whereas hexane extracts had the least bactericidal potential.	Hengameh and Rajkumar (2017)

(continued)

Table 15.1 (continued)

Name of Lichens/metabolites	Tested against Bacteria	Results	Reference
Eleven natural lichen compounds viz., Methyl-beta-orcinolcarboxylate, Psoromic acid, Conhypoprotocetraric acid, Demethylbarbatic acid, Hypoprotocetraric acid, Variolaric acid, Vulpinic acid, (+)-Erythrin, Lepraric acid, (+)-acetylportentol, (+)-Roccellic acid	*Streptococcus gordonii* and *Porphyromonas gingivalis*	Three compounds showed promising antibacterial activities where the depsidone core with certain functional groups constituted the best compound, psoromic acid, with the lowest MICs = 11.72 and 5.86 µg/mL against *S. gordonii* and *P. gingivalis*, respectively	Sweidan et al. (2017)
Ramalina capitata acetone extract	Two Gram-positive (*Bacillus spizizenii* and *Staphylococcus aureus* and three Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella abony*)	Maximum inhibition zone was 28 mm for *Bacillus spizizenii,* Weaker antibacterial activity of the extract was for *Staphylococcus aureus*, with zone inhibition 19 mm	Ivana et al. (2017)
Ramalina species viz. *R. hossei* Vain, *R. conduplicans* Vain and *R. pacifica Asahina*	*B. cereus and E. coli*	*R. pacifica* and *R. hossei* inhibited bacteria to the highest and least extent respectively	Ankith et al. (2017)
Ethyl acetate Extracts of *Parmelia vagans*	*Staphylococcus aureus, Bacillus subtilis, and Streptococcus pneumoniae.*	Complete inhibition of growth was observed	Bondarenko et al. (2017)
Methanol, acetone and chloroform extracts of the lichen, *Usnea florida*	*Bacillus cereus, Bacillus subtilis, Escherichia coli, Enterococcus faecalis, Enterobacter aerogenes, Klebsiella pneumoniae, Listeria monocytogenes, Micrococcus luteus, Mycobacterium tuberculosis, Pseudomonas aeruginosa, Proteus vulgaris Staphylococcus aureus Salmonella typhimurium and Yersinia enterocolitica*	Maximum sensitivity to the species examined was demonstrated by *Bacillus cereus* and *Bacillus subtilis* with an inhibition zone of 20 mm	Cankılıç et al. (2017)
Organic extract of *Cladonia substellata* Vainio and usnic acid	*Staphylococcus* spp. derived from samples of skin and ears of dogs and cats	Antimicrobial activity *in vitro* ranged from 0.25 mg/ml to 0.0019 mg/ml inhibiting bacterial growth at low concentrations	Moura et al. (2017)

Ethyl acetate extracts of *Flavoparmelia caperata, Usnea longissima Ramalina conduplicans* and *Everniastrum cirrhatum*	Clinical strains of *Escherichia coli Pseudomonas aeruginosa, Salmonella typhimurium, Bacillus subtilis* and *Klebsiella pneumoniae*	*U. longissima* and *F. caperata* showed significant activity against all the bacteria but *U. longissima* effective against *Escherichia coli* (13.0 mm; MIC=7.5 mg/ml) and *Bacillus subtilis* (11.6 mm; MIC = 7.5 mg/ml) while *F. caperata* active against *E. coli* (15.3 mm; MIC = 15 mg/ml).	Kumar et al. (2017)
Heterodermia incana (Stirt.) metabolites such as zeorin and atranorin	Gram-positive bacteria (*Bacillus subtilis* and *Bacillus cereus*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*)	Showed inhibitory activity against *Bacillus cereus* (zone of inhibition 2.26 ± 0.05 cm) and *Pseudomonas aeruginosa* (zone of inhibition 1.76 ± 0.05 cm)	Kekuda et al. (2017)
Fruticose lichen genera *Ramalina* and *Usnea*	*Staphylococcus aureus, Bacillus subtilis*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*	Both Methicillin-resistant *Staphylococcus aureus* (**MRSA**) and *S. pneumoniae* inhibited up to 18mm each, crude extracts exhibited promise *in vitro* against the selected bacteria, since the ZOI was significantly higher than that of the positive control Vancomycin (30mg/ml, mean difference = 7 mm, sig. α =.05).	Timbreza et al. (2017)
Protoparmeliopsis muralis, Caloplaca pusilla and *Xanthoria parietina*	Gram-positive bacteria i.e. *Bacillus subtilis*, *Enterococcus faecalis, Staphylococcus aureus* and *Staphylococcus epidermidis*	*P. muralis* extract strongly inhibited the growth (MICs from 6.67 to 100.00 μg ml^{-1})	Felczykowska et al. (2017)

(continued)

Table 15.1 (continued)

Name of Lichens/metabolites	Tested against Bacteria	Results	Reference
Methanol, ethanol, chloroform, acetone and aqueous extracts of the lichen *Parmotrema tinctorum* and *Pyxine sorediata*	Seven pathogenic bacteria (*Klebsiella pneumoniae, Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella enterica ser. typhi, Escherichia coli* and *Shigella boydii*)	Acetone and ethanol extract of *Pyxine sorediata* showed potential antibacterial activity (ZOI, 8–13 mm) followed by chloroform and methanol extracts (ZOI, 7–9 mm), acetone and ethanol extracts of *Parmotrema tinctorum* showed 3–9 mm zone of inhibition respectively	Swamy et al. (2016)
Acetone extract *of Usnea rubrotincta, Ramalina dumeticola* and *Cladonia verticillata*	*Staphylococcus aureus* and *Bacillus subtilis*	Promising antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus,* MIC (15.63 µg/ml) for the *U. rubrotincta* against *B. subtilis*. *C. verticillata* extract was neither active against gram positive nor gram negative bacteria at the highest tested concentration tested@ 500 µg/ml.	Saranyapiriya et al. (2015)
DMSO, aqueous extracts, Ethanolic, isopropanolic, acetone of *Usnea* sp., *Ecuadorian highland* and *Stereocaulon* sp.	*Bacillus subtilis, Escherichia coli* and *Staphylococcus aureus*	Showed inhibition zones; 28 mm, 30 mm, 31 mm (DMSO extract, ciprfloxacin and cefazolin respectively) against *B. subtilis*	Matvieieva et al. (2015)
Methanol and acetone extracts of four lichens viz., *Parmotrema praesorediosum*, *P. rampoddense*, *P. tinctorum* and *P. reticulatum*	*Staphylococcus aureus* and *Bacillus subtilis*	Acetone extracts resulted good inhibitory activity against *Bacillus subtilis and Staphylococcus aureus* and with MIC values ranging from 125 to 500 µg/ml, but methanol extracts had no activity	Rajan et al. (2015)

34 lichen species mainly viz., *Cladonia furcata, Bryoria fuscescens, Alectoria imshaugii, Evernia prunastri, Flavocetraria nivalis, Letharia columbiana*, *L. vulpina*, and *Vulpicida canadensis* etc	Against *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli*	Showed inhibitory against *Pseudomonas aeruginosa* and *Staphylococcus aureus, S. aureus* with MIC values ranging from 3.9 to 500 µg/ml.	Shrestha et al. (2014)
Ramalina conduplicans petroleum ether, ethyl acetate and ethanol Solvent extracts	against 15 bacteria	Showed dose dependent inhibitory activity and least susceptibility was for *E. faecalis* and *K. pneumoniae* while *E. faecalis* and *P. aeruginosa* inhibited to high extent	Kambar et al. (2014)
Ethanolic extract of *Parmelia sexatilis, P. caperata, P. parletum, Everniastrum cirrhatum, Parmelia pereoridisum, Parmoterma mesotropum, P. reticulatum, P. perlatum, and Parmelia squarrosa*	*Staphylococcus aureus*, *Mycobacterium smegmatis* and *Micrococcus luteus*, *Bacillus cereus*	Extracts of *Parmelia caperata* (8–32 µg/ml MIC), *P. parletum* (16–32 µg/ml MIC), *P. sexatilis* (16–32 µg/ml MIC) and *Everniastrum cirrhatum* (8–32 µg/ml MIC) showed maximum activity	Kumar et al. (2014)
Aqueous extract of *Parmotrema* sp.	Bacteria viz., *Salmonella* sp., *Shigella* sp., *Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Enterococci faecalis, Klebsiella pneumonia, Pseudomonas aeruginosa,*	Showed antibacterial activity against *Staphylococcus aureus, Pseudomonas aeruginosa* but weak activity against *Salmonella* sp. and *Scedosporium* sp.	Chauhan and Abraham (2013)
Usnic acid	20 different methecillin resistant clinical isolates of *S. aureus*	Usnic acid inhibited 50% of growth of all *S. aureus* at 2 µg/ml while 90% inhibition at 4 µg/ml.	Segatore et al. (2012)
Acetone-, methanol- and aqueous-extracts of the lichens *Lecanora atra, L. muralis, Parmelia saxatilis, P. sulcata* and *Parmeliopsis ambigua*	*Bacillus mycoides* (IPH), *B. subtilis* (IPH), and *Staphylococcus aureus* (IPH) (Gram-positive bacteria); and *Enterobacter cloacae* (IPH), *Escherichia coli* (IPH) and *Klebsiella pneumoniae* (IPH), (Gram negative bacteria)	Strongest antimicrobial activity found in the acetone extract of *Parmelia sulcata* where the least MIC was 0.78 mg/ml.	Kosanic and Rankovic (2011)

(continued)

Table 15.1 (continued)

Name of Lichens/metabolites	Tested against Bacteria	Results	Reference
Essential oils of *Evernia prunastri* and *Evernia divaricata*	*Escherichia coli, Yersinia pseudotuberculosis, Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis, Bacillus cereus*	*E. divaricata* oil exhibited the antimicrobial activity	Kahriman et al. (2011)
Umbilicaria cylindrical crude extract	*K. pneumonia, S. aureus, B. subtilis E. coli, Proteus vulgaris P. mirabilis*	Ethyl acetate showed potent activity against *E. coli* with MIC 15.62 µg/ml but methanolic extract showed activity against *B. subtilis* and *S. aureus* having MIC value 15.62 µg/ml	Manojlovic et al. (2012)
Various orsellinic acid esters, depsidones depsides, xanthones, usnic acid, lichexanthone derivatives and salazinic acid derivatives of *Pseudoparmelia sphaerospora Parmotrema tinctorum P. dilatatum, P. tinctorum, Usnea subcavata*	*Mycobacterium tuberculosis*	Inhibited growth of *M tuberculosis,* diffractaic acid (MIC = 15.6 µg/ml) was most active and norstictic acid (MIC = 62.5 µg/ml), hypostictic acid (MIC = 94.0 µg/ml), usnic acid (MIC = 62.5 µg/ml) and protocetraric acid (MIC = 125 µg/ml) respectively	Honda et al. (2010)
Stictic acid barbatic acid Usnic acid, norstictic acid, salazinic acid and galbinic acid from diffractaic acid *Ramalina dendriscoides, Usnea baileyi, Cladonia gracilis* and *Stereocaulon massartianum*	*B. subtilis S. aureus E. coli P. aeruginosa*	effective against *B. subtilis* and *S. aureus and* but moderately active against *E. coli* and *P. aeruginosa, R dendriscoides,* and highest activity against *S. aureus* with MIC and MBC values of 156 and 2,500 µg/ml	Santiago et al. (2010)
Barbatic acid extracted from *Cladia aggregata*	Against *S. aureus* strains	Checked the growth of *S. aureus,* showed MIC values against different strains of *S. aureus* 50 µg/ml	Martins et al. (2010)
Usimine A, Usnic acid, Usimine B, Usimine C extracted from *Ramalina terebrata*	*P. aeruginosa, B. subtilis, E. coli, S. aureus*	Usnic acid showed highest MIC value of 1.2 µg/mL.	Paudel et al. (2010)

Divaric acid, 2-O-methylnordivaricatic acid, 2,4-di-O-methyldivaric acid, usnic acid, divaricatinic acid of *Evernia divaricata*	*Bacillus subtilis S. aureus E. coli P. aeruginosa*	Prouced large inhibition zone for *B. subtilis, S. aureus* and *P. aeruginosa,* usnic acid showed MIC value of 0.0078 mg/ml against *B. subtilis* while divaric produced inhibitory action having MIC value 0.625–0.25 mg/ml against all four bacteria	Yuan et al. (2010)
Xanthoria parietina *Xanthoria elegans* *Xanthoparmelia tinctina* *Umbilicaria vellea* *Rhizoplaca melanophthalma* *Ramalina farinacea* *Peltigera praetextata* *Peltigera polydactyla* *Lecanora muralis* *Cetrelia olivetorum* *Anaptychia ciliaris* crude extracts	*B. subtilis* *E. coli* *K. pneumoniae* *P. aeruginosa* *S. aureus* *Staphylococcus*	Showed inhibition against *B. subtilis, S. aureus* and *E. coli* but ethanol extracts of some lichens showed activity against *B. subtilis, S. aureus* and *S. epidermidis.*	Karagoz et al. (2009)
Gyrophoric acid (*U. polyphylla*) Physodic acid (*H. physodes*), usnic acid (*Parmelia caperata*), atranorin (*Physcia aipolia*)	*S. aureus* *K. pneumoniae* *Enterobacter cloacae* *E. coli* *B. subtilis* *B. mycoides*	Usnic acid of the *Parmelia caperata* lichen showed lowest MIC 0.0037 mg/ml against the *Klebsiella neumoniae* while weakest activity reported in physodic acid, which inhibited most of the microorganisms in the concentration of 1 mg/ml.	Rankovic et al. (2008)

(continued)

Table 15.1 (continued)

Name of Lichens/metabolites	Tested against Bacteria	Results	Reference
3-hydroxyphysodic acid (*H. physodes*) Evernic acid (*Evernia prunastri*) physodic acid (*H. physodes*) salazinic acid (*Flavoparmelia caperata*) atranorin (*Lepraria lobificans*) Hybocarpone (*Lecanora conizaeoides*) lobaric acid (*Sterocaulon dactylophyllum*) physcion(*X. parietina*) rhizocarpic acid (*Psilolechia lucida*) usnic acid (*Lecanora albescens*)	*S. aureus*	Hybocarpone was effective against all with an MIC at 4–8 µg/ml but evernic acid and atronorin showed activity against *S. aureus*-1199B strain only	Kokubun et al. (2007)
Acetone extract of *R. melanophthalma* *R. peltata* *Rhizoplaca chrysoleuca*	*B. megaterium* *B. subtilis* *E. coli* *Enterococcus faecalis* *Pseudomonas mirabilis. P. aeruginosa* *S. aureus*	Zone of inhibition of *R. chrysoleuca* was greater than antibiotic tetracyclin in the case of *E. coli, P. mirabilis* and *B. megaterium* *R. chrysoleuca* showed high activity against all bacteria except *P. aeruginosa, R. melanophthalma* extract showed activity against only	Cansaran et al. (2006)
Extracts of *Umbilicaria nylanderiana* *Ramalina polymorpha* *Ramalina pollinaria* *Platismatia glauca* *Parmelia saxatilis*	35 strains of bacteria belonging to genera viz., *Shigella, Staphylococcus, Xanthomonas, Yersinia Acinetobacter, Cedecea, Enterobacter, Escherichia, Klebsiella, Morgonella, Bacillus, Brucella, Burkholdria, Clavibacter Proteus, Pseudomonas, Salmonella, Serratia, Streptococcus,*	*R. pollinaria* checked 11 bacterial species out of 35 bacteria tested	Gulluce et al. (2006)
Usnic acid	*P. aeruginosa* *S. aureus*	Inhibited through formation of the bio-film by killing the attached cells	Francolini et al. (2004)

Atranorin (*Stereocaulon alpinum*) Lobaric acid (*S. alpinum*) salazinic acid (*P. saxatilis*) protolichesterinic acid (*Cetraria islandica*) Usnic acid (*Cladonia arbuscula*)	*Mycobacterium aurum*	Usnic acid showed an MIC 32 µg/ml but were inactive at these dose	Ingolfsdottir et al. (1998)
Usnic acid (*Cladonia stellaris*) Usnic acid (Commercial) vulpinic acid (*Letharia vulpine*)	*Bacteroides vulgatus, Bacteroides fragilis, Pseudomonas aeruginosa, Candida albicans, Saccharomyces cerevisiae, Clostridium perfringens, Bacteroides thetaiotaomicron, Escherichia coli, Enterococcus faecalis, Haemophilus influenza, Staphylococcus aureus, Streptococcus pneumoniae*	Inhibited the growth of *S. aureus, E. faecalis, E. faecium* and some anaerobic species (*Bacteroides* and *Clostridium* species) at the concentrations tested, Vulpinic showed less activity than usnic acid.	Lauterwein et al. (1995)
Alectosarmentin from *Alectoria sarmentosa* usnic acid, physodic acid, ethyl-palectoronic	*E. coli* *K. pneumoniae* *P. aeruginosa, S. aureus* *Salmonella gallinarum*	Isolated acids wee active against *K. pneumoniae* and *P. aeruginosa*	Gollapudi et al. (1994)
17 species of lichens	*S. aureus* *P. aeruginosa*, *E. coli* *B. subtilis*	Active against gram-positive and negative as well.	Ingolfsdottir et al. (1985)

15.5 Promising Sources of Biotechnologically Important Bacteria

The search for microorganisms from novel sources and in particular microbial symbioses represents a promising approach in biotechnology. For this lichens are now becoming a subject of microbial biotechnology research due to recognition of diverse community of bacteria other than cyanobacteria. The lichens can harbor diverse bacterial families useful for the production of compounds of biotechnological interest. Microorganisms need to be isolated from lichens in particular Actinobacteria and Cyanobacteria which can produce a number of bioactive compounds with biotechnological potential (Suzuki et al. 2016).

15.6 Genetic Approaches for Harvesting Products from Lichen

The molecular genetic techniques such as heterologous expression, PCR and genomic library construction is now becoming an alternative tool for investigating the polyketide biosynthetic pathways in lichens. The techniques can be integrated with conventional culture collection based programme of screening for providing a comprehensive approach for new chemical molecules in Lichens (Miao et al. 2001).

Now a interest showing popularity to search a low molecular weight molecules in lichens for polyketides drugs. These Polyketides were studied through mass spectrometry and NMR (Su et al. 2003). New synthetic thought may create engineering in structurally diverse compounds (Seitz and Reiser 2005). Kristmundsdóttir et al. (2005) have studied how to enhance the water solubility in depsides and depsidones in common pharmacological models. While Esimone et al. (2005) investigated vector based assay to screen bioactive compounds present in lichens. Cloning, genetic engineering and homologous or heterologous expression may hit natural products production in lichens (Miao et al. 2001). But there was no expression of lichen ketides successful on prokaryotic as well as eukaryotic hosts (Brunauer and Stocker-Wörgötter 2005). DOE Joint Genome Institute-US Department of Energy plans to sequence genome of *Xanthoria parietina* which is the first lichen fungus. These genome sequences of lichens will speed up genetic and proteomic methods.

15.7 Optimization of Culture Conditions

In order to study culture conditions in lichen *Usnea ghattensis* the experiments were done to increase biomass and antioxidant metabolite production. They were Cultured on growth media having excess carbon contents. This increased total polyphenol mass fraction, usnic acid production and biomass after 6 months of inoculation. The methanol extract of 6-month-old cultures grown in the malt yeast extract (MYE) medium having 0.01 mol/L of sucrose and 0.01 mol/L of polyethylene glycol resulted inhibition of lipid peroxidation activity significantly up to 89% (Bhaskar et al. 2009).

15.8 Conclusions

Compounds present in Lichens are useful for pharmaceutical purposes but some properties of lichens still needs for consideration. They have slow growth both in ecosystem and in axenic cultures is a major problem in the production of metabolites. But culture conditions of lichen mycobionts may be optimized to support for synthesis of interesting secondary compounds having medicinal value.

The lichens have valuable antibacterial potential. Whose formulations or are useful in treating many infectious diseases. They are reservoirs of low molecular weight secondary compounds. A few compounds only have been studied hence it urgently needs continued screening programmes across their diversity area. It needs more deep studies for compounds that have already shown promise against pathogenic bacteria. This in turn needs more clinical trials as well as commercial production with effective implementation of drug lines.

The new bioactive molecules obtained from Lichens will be able to replace synthetic additives. Also they can be effectively used in the food and pharmaceutical industries. The literature clearly reveals that the lichenized fungi(extracts)possess significant antibacterial activity. They can also be a good source of antibiotics and secondary metabolites. Therefore, it can be concluded that more in vivo studies are needed on priority to check their broad-spectrum bactericidal activity.

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Symbiosis Between Sebacinales and *Aloe vera*

16

Priyanka Sharma, Monika Tyagi, Amit Kharkwal, Cristina Cruz, and Ajit Varma

Abstract

Role of soil microflora is important for survival growth and adaptation of plants in diverse habitat. Mutualistic microorganisms especially mycorrhiza play a key role in adaptation of plants against biotic and abiotic stress under challenging environmental conditions. Mycorrhizal fungi belonging to the order sebacinales are distinguished by the fact that they are culturable and have significant biotechnological prospects in making plants tolerant to stress. Safe, predictable and sustainable cultivation of medicinal plants are a must for industry to make quality herbal products for benefits of consumers. Cultivation of medicinal plants holds significance in restoration of degraded lands using sebacinales fungi as they provide source of income to marginal farmers. *Aloe vera* is a highly valued plant for its medicinal and cosmetic applications since time immemorial. In this study micropropogated *Aloe vera* plants colonized with sebacinales fungi Piriformospora indica survived better under the influence of NaCl at moderate (100 mM), high (200 mM) and severe (300 mM) stress. The treated plants showed better growth, higher antioxidant scavenging activity and production of secondary metabolites.

Keywords

Arbuscular mycorrhizal fungi · *Piriformospora indica* · Symbiosis · *Aloe vera* · Salt stress · Secondary metabolite

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16.1 Introduction

16.1.1 Mutualistic Symbiosis Between Fungi and Autotrophs

A range of micro organisms such as algae, bacteria and fungi constitute soil micro flora. They actively contribute in almost all the chemical processes that occur within the soil. They participate in nutrient acquisition, environment cycles, tolerance to biotic and abiotic stresses and various other processes that play a crucial role in survival and growth of the plant. In contrast, microbes colonizing the rhizospheric region get benefitted from the plant, i.e., increased availability of carbon in the soil, which supplement their growth (Barea et al. 2005). On the other side, plants being the immobile, are confronted with a number of unfavourable abiotic and biotic conditions, e.g., drought, salinity, pathogen attacks etc. However, with the course of evolution, to protect themselves from the attack of such stresses, they have developed a number of protective mechanisms (Kogel et al. 2006). The best strategy is to form a mutualistic symbiotic association with beneficial microorganisms (Lum and Hirsch 2003).

The importance of such mutualistic association lies in increased nutrient availability to the plant, improved rhizospheric and soil conditions, with better growth and enhanced vigour of the plant and availability of carbon source to the fungus in the form of amino acids, sugar and vitamins essential for its growth. This ultimately results in the dilution of toxic ion effect (Sharma et al. 2015).

16.2 Arbuscular Mycorrhizal Fungi

A vast majority of temperate and tropical plant species grow in the field under the influence of mutualistic association with fungi. Among such associations, the most widespread, geographically as well as within the plant kingdom, are the Arbuscular Mycorrhizal (AM) Fungi (Varma 1999). This association is a keystone to the productivity and diversity of natural plant ecosystems. These are associated with the roots of around 80% terrestrial plant species, which includes xerophytes, halophytes and hydrophytes. This is a mutualistic symbiotic association because of the highly beneficial relationship between both partners. The host plants receive mineral nutrients via the fungal mycelium, while the fungi, which is heterotrophic, obtain carbon nutrients from the host plants (Varma et al. 1999, 2004, 2017a; Harley and Smith 1983). Arbuscular mycorrhizal (AM) fungi belong to nine genera: *Gigaspora, Glomus, Acaulospora, Scutellospora, Archaeospora, Gerdemannia, Entrophospora, Paraglomus and Geosiphon*, the only known fungal endosymbiosis with cyanobacteria (Schuessler and Kluge 2001; Schuessler 2009).

These fungi colonize the roots and rhizosphere and branch more frequently and extensively in comparison to the plant roots, hence forming a ramified network which increases the absorptive capacity of roots and thus better access to greater

quantity of water and minerals, which are required for nutrition. Thus the most prominent and consistent function of the AM fungi is to enhance the uptake of nutrients, especially those whose diffusion is limited, such as P, Cu, K, Zn and Fe. Their role becomes even more crucial in areas where, fertilization of extensive land areas with large quantities of phosphorus is not practical, due to its unavailability or its high cost (Sharma et al. 2015).

Arbuscular mycorrhizal fungi acquire their name from their distinctive arrangement of highly branched structures called *Arbuscules*, present inside the cortical cells of roots (Bever et al. 2001). AM fungi develop symbiotic association with almost 80% of plants, they enhance the contact between fungus and plant root (Varma et al. 2017a). Arbuscular Mycorrhizal Fungi are obligate biotrophs and use the carbon source (photosynthate) of plant for survival and reproduction. Thus AM fungi play an important role in sequestration and decomposition of soil carbon (Varma et al. 2017b).

16.3 Breakthrough Research

AM fungi are noncultivable on synthetic medium (axenically). Ajit varma and his collaborators discovered the root-colonizing symbiotic fungus *Piriformospora indica* (*Serendipita indica*) from the rhizosphere of the woody shrubs *Prosopsis juliflora* and *Zizyphus nummularia*. *P. Indica* was grouped under Hymenomycetes (Basidiomycota), based on the ultra structure of hyphae (presence of dolipore septa) and 18s-rRNA gene sequence. It has a similar appearance to AM Fungi which, however comes under a new family *Sebacinaceae* (Weiß et al. 2004). Based on nuclear rDNA sequence for the 5′ terminal domain of the ribosomal large subunit, the fungus was placed as a member of new order *Sebacinales*. Fungus infests roots of a broad range of mono- and dicotyledonous plants. This new order branched into two suborders: group A or *Sebacinaceae* and group B *Serendipitaceae* (Weiß et al. 2016), (Fig. 16.1). *P. indica* can easily be cultured without a host plant, thus it is a suitable model system to study plant–microbe interactions. The *P. indica* colonization like other mutualistic endophytes is in an asymptomatic manner. The root colonization by *P. indica* gradually increases with tissue maturation, as revealed by PCR-based quantification. No colonization is shown by the root tip meristem, however the elongation zone shows mainly intercellular colonization. The inter- and intracellular hyphae and intracellular chlamydospores heavily infests the differentiation zone. The epidermal and cortical tissues get completely filled with chlamydospores, thought he fungal mycelium never reaches the stellar tissue. The *P. indica* has a broad range of hosts, which includes, Bryophytes (*Aneura pinguis*), Pteridophytes (*Pteris ensiormis*), Gymnosperms (*Pinus halepensis*), and a large number of Angiosperms including as diverse as economically important crops, medicinal, forests, orchids and ornamental plants.

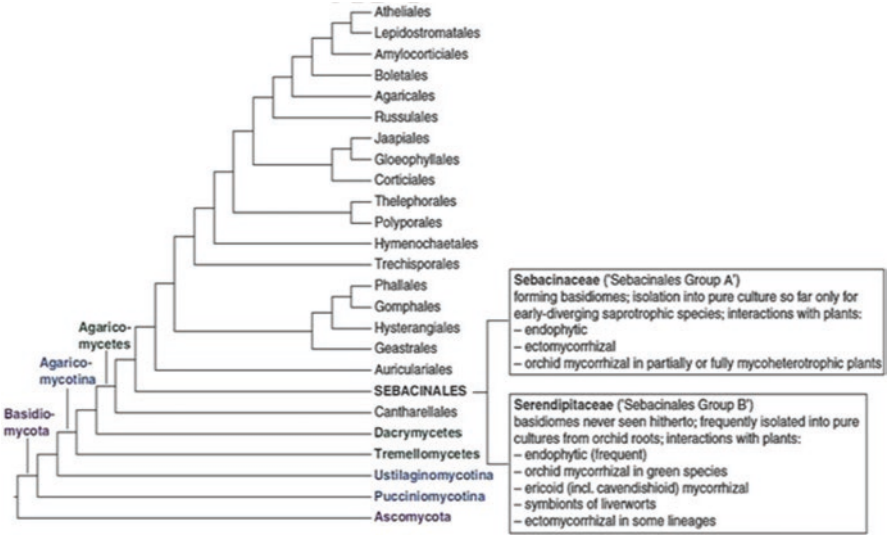

Fig. 16.1 Sebacinales: phylogenetic position within Basidiomycota and interactions of the two families (Sebacinaceae and Serendipitaceae) with plants. (Weiß et al. 2016; Singhal et al. 2017)

16.3.1 Functions Involved

It is a well established fact that *P. indica* mimics the capabilities of typical AM fungus and improves the growth and overall biomass production. It has a broad spectrum of hosts, including herbaceous mono- and dicotyledons, and trees, including medicinal plants, and several economically important crops. Pronounced growth promotional effect was also seen with terrestrial orchids. Among the compounds released in root exudates, flavonoids are found to be present in *P. indica*. They are thought to be involved in stimulation of pre-contact hyphal growth and branching, which suggests their role as signalling molecules in other plant-microbe interactions. Also, the enzymes which degrade the cell wall like carboxymethyl cellulase (CMCase), xylanase and polygalactouronase were found in significant quantities both in the culture filtrate and in the roots colonized with *P. indica*.

16.4 Symbiosis of *Sebacinales*

The environmental stresses causes either death of the plant or decrease in productivity, which correlates well with the damaging effects of oxidative stress. Oxidative stress leads to the generation of free oxygen radicals or Reactive Oxygen Species (ROS), which are cytotoxic, at high concentrations. For uninterrupted growth and survival of plants, there is a need for an efficient scavenging system, that can fight against the deleterious effects of ROS. The plants thus are endowed with enormous antioxidants and metabolites in different plant cell compartments (Ashraf and Harris 2004; Sharma et al. 2014b) (Fig. 16.2).

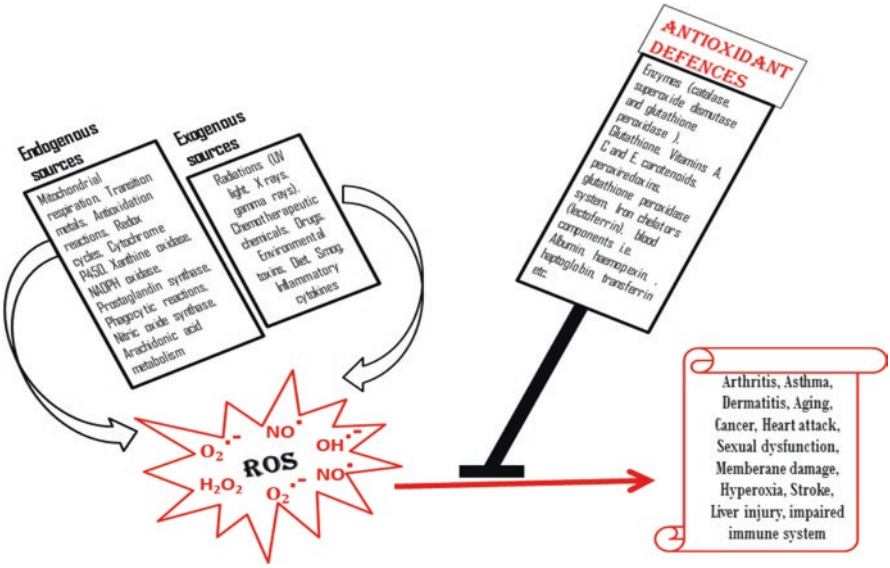

Fig. 16.2 Role of antioxidants in scavenging free radicals that cause a number of diseases. ROS stands for reactive oxygen species. (Sharma et al. 2014b)

The plants have evolved with both nonenzymatic and enzymatic scavenging mechanisms to fight against deleterious effects of free oxygen radicals. These two are called as Antioxidant Defence Systems. Salinity stress affects a number of processes involved in salt tolerance mechanism, including a number of compatible solutes or osmolytes, polyamines, ion transport, compartmentalization of toxic ions, reactive oxygen species and antioxidant defence mechanism (Sairam and Tyagi 2004; Ashraf and Harris 2004).

These *Sebacinales* colonize roots as endophytes. Fungi of this distinct order *Sebacinales* are involved in a broad variety of mutualistic associations with various plants, thus they possess potential to act as biocontrol agents. It is able to amplify biomass and yield of crop plants and has enormous potential for sustainable agriculture. This relation however requires a balance between the defense responses of the plant and the nutrient demand of the fungus. *Sebacinales* strains may be perfect models for the study of valuable fungus-plant communications and have a promising outlook for application in sustainable horticulture and agriculture (Deshmukh et al. 2006).

Growth promotional characteristics of *P. indica* have been studied in a great number of plants and most of them have shown highly significant outcomes, which necessitates the need for its mass cultivation. Thus, a formulation called "Rootonic" has been prepared by mixing *P. indica* biomass in magnesium sulfite (raw talcum powder). The quantity of formulation to be used per hectare of land for maximum productivity has also been standardized for about 150 plants (Singhal et al. 2017). There has been a growing focus on the importance of medicinal plants and their extracts in solving the health care problems of the world. Such cultivation system has shown to remediate salinity as well as provide an income to resource poor farmers.

Plants being immobile are confronted with a number of unfavourable conditions, like drought, salinity, pathogen attacks, etc. However, with the course of evolution, to protect themselves from the attack of such stresses, they have developed a number of protective mechanisms (Kogel et al. 2006). Hence, in order to protect themselves from such stresses, the best strategy is to form a mutualistic symbiotic association with beneficial microorganisms (Lum and Hirsch 2003). However, the most difficult task is to differentiate a mutualistic partners with a parasites (Kogel et al. 2006; Schulz and Boyle 2005), as both of these interactions share a number of common signalling pathways (Paszkowski 2006). The symbioses can have an array of ecological and evolutionary importance, such endophyte plant interaction and mycorrhiza plant interaction.

16.5 *Aloe vera* the Plant of Health, Wealth, Economic Viability and Industry

16.5.1 Morphology, Taxonomy and Biotechnological Application of *Aloe vera*

Plants have been used as an important source of medicine for thousand of years. Even today, around 80% of world population still rely on traditional medicines, as per WHO estimates (Yates 2002). The genus *Aloe* is a perennial, succulent xerophytic plant grown in temperate and sub-tropical parts of the world. It has originated from Africa. *Aloe vera* or *Aloe barbadensis* belongs to the Asphodelaceae family, of which there are over 360 known species (Vogler and Ernst 1999). There are several species under the genus *Aloe*, including *Aloe vera, Aloe barbadensis, Aloe indica, Aloe ferox, Aloe chinensis, Aloe peyrii* etc. amongst these, *Aloe vera* Linn syn. *Aloe barbadensis* Miller (Chandegara and Varshney 2013). It is commonly called 'aloe', burn plant, lily of the desert and elephant's gall. It is a cactus-like plant with green, fleshy leaves, filled with clear viscous jel and ends are tapering and have spines (Cheesbrough 2000; Joseph and Raj 2010; Manvitha and Bidya 2014).

It has a very short stem that grows up to 80–100 cm. The leaves of Aloe have adapted to grow in dry areas of low rainfall, by becoming thick and fleshy. Leaves vary in colour from green to grey-green, with a serrated margin. Flower is pendulous, with a yellow tubular corolla, and are produced on a spike upto 90 cm tall (Kumar et al. 2010).

Aloe has great demand in industry, cosmetics and pharmaceutical sector, so its cultivated in large quantities (Figs. 16.2 and 16.3). The pharmacological actions of *Aloe vera* include anti-inflammatory, anti-arthritic, antibacterial activity and hypoglycaemic effects. It is called the healing plant because of its wound and burn healing properties (Choi and Chung 2003). *Aloe vera* has been used for medicinal purposes in several countries for millennia, such as Greece, Egypt, Mexico, Japan, India and China (Marshall 1990). The bio active compounds of *Aloe* are used as antidiabetic, antiulcer, anti-septic, antibacterial, anti-inflammatory, antioxidant and anticancer agent. They have also proven effective in treating various ailments, such

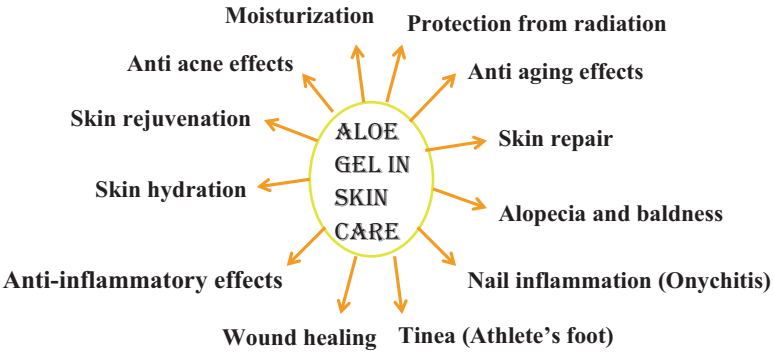

Fig. 16.3 Uses of Aloe gel in skin care. (Sharma et al. 2014b)

as gastrointestinal problems, constipation, radiation injury, wound healing, burns, dysentery, and diarrhoea and in the treatment of skin diseases (Joseph and Raj 2010; Yongchaiyudha et al. 1996; Bunyapraphatsara et al. 1996; Hirat and Suga 1983; Rabe and Staden 1997).

Aloe vera is called *Ghrit Kumari* in "Sanskrit". It has been described in Ayurveda as a multi-functional herb, as a blood purifier, anti- inflammatory, diuretic, uterine tonic, laxative and fever reliever. It has also been used in Ayurveda for appetite-stimulant, purgative, and for cough, cold, piles, asthma and jaundice (Joseph and Raj 2010). *Aloe* is used in a wide variety of products including cosmetics, creams and toiletries (Dat et al. 2012; Sharma et al. 2014b; Steenkamp and Stewart 2007) (Fig. 16.3).

It has been used for the production of health drinks and beverages in a number of food industries. In the cosmetics and toiletry products, it is used as base material for the production of creams, lotions, soaps, shampoos, facial cleansers and other products. It is used for the manufacturing of topical products such as ointments and gel preparations, as well as in the production of tablets and capsules, in Pharmaceutical industries (Eshun and He 2004; He et al. 2005; Hamman 2008). The gel obtained from Aloe may be used to effectively deliver poorly absorbable drugs through the oral route of drug administration, due to its absorption enhancing effects. Hence, dried aloe gel has been successfully used to manufacture directly compressible matrix type tablets (Jani et al. 2007; Hamman 2008).

16.6 Chemical Composition of *Aloe* Gel

The chemical composition of the *Aloe vera* gel is complex. It contains 75 potentially active constituents such as vitamins, sugars, enzymes, minerals, lignin, salicylic acids and amino acids (Fig. 16.5) (Atherton 1998; Vogler and Ernst 1999). The detail is as follows:

Vitamins: The plant contains many vitamins, including Vitamins A, C and E, which are antioxidants. It also contains thiamine, niacin, riboflavin, vitamin B12, cho-

line and folic acid (Coats 1979; Vogler and Ernst 1999). Antioxidant neutralizes free radicals.

Enzymes: Amylases, lipases, alkaline phophatases, cellulases, catalases and peroxidases are biochemical catalysts that help in digestion by breaking down fats and sugars. Carboxy peptidases and bradykinases, produce an anti-inflammatory effect by inactivating bradykinins (Surjushe et al. 2008). Lectins give anti-tumour effects (Kumar et al. 2010).

Minerals: Sodium, potassium, calcium, magnesium, selenium, manganese, copper, zinc, chromium and iron are all found in the aloe plant. These minerals play an important role in functioning of enzymes, involved in various metabolic pathways. Few of these, act as antioxidants (Surjushe et al. 2008).

Sugars: Sugars are located in the mucilaginous layer of the plant under the rind of the leaf. It includes monosaccharides (glucose and fructose) and polysaccharides (glucomannose and polymannose). The polysaccharides act as immune modulators (Green 1996; Kumar et al. 2010). Glumannan is a good moisturizer and used in cosmetic products (Chandegara and Varshney 2013).

Anthraquinones: The bitter reddish yellow exudates, located beneath the outer green rind, contains anthraquinones and their derivatives, aloe-emodin-9-anthrone, lsobarbaloin, Anthrone-C-glycosides, Barbaloin and chromones. These are phenolic compounds, traditionally known as laxatives. These compounds exert a powerful purgative effect, when in large amount, but when smaller they appear to aid absorption from the gut and are potent antimicrobial agents and possess powerful analgesic effects (Joseph and Raj 2010).

Sterols: These include cholesterol, campesterol, β-Sitosterol and lupeol. They have anti- inflammatory action and lupeol also possesses antiseptic and analgesic properties (Coats 1979; Surjushe et al. 2008).

Hormones: Auxins and gibberellins that help in wound healing and have anti-inflammatory action.

Salicylic acid: This is an aspirin-like compound possessing anti-inflammatory and antibacterial properties.

Amino acids: *Aloe vera* gel provides the amino acids required for repair and growth. It includes 20 of 22 non- essential amino acids and 7 of 8 essential ones (Joseph and Raj 2010).

Lignin: It is an inert substance. It enhances penetrative effect of various ingredients into the skin when included in topical preparations (Surjushe et al. 2008).

Saponins: These are the soapy substances that have cleansing and antiseptic properties (Surjushe et al. 2008).

16.7 Secondary Metabolites in *Aloe vera*

Aloe leaf consists of two parts (Fig. 16.4). The inner parenchymal tissues that form a clear, thin, tasteless, jelly-like material (Tarro 1993) and the outer pericyclic tubules, that occur just beneath the outer green rind of the leaves and produce bitter yellow exudates (Vogler and Ernst 1999; Sharma et al. 2014b). The inner

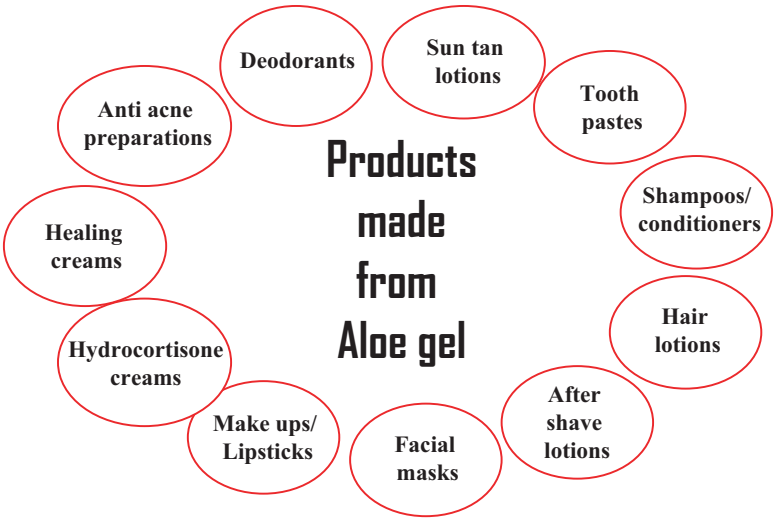

Fig. 16.4 The picture depicts various products made from Aloe gel. (Sharma et al. 2014b)

mucilaginous pulp called *Aloe* gel lies in the centre of leaf. The Aloe gel contains around 96% water while the remaining 4% consists of 75 known substances including Vitamins A, B, C, and E, calcium, amino acids and enzymes (Sharma et al. 2014b; Joseph and Raj 2010) (Fig. 16.5). The polysaccharides present in the inner parenchymatous tissue are thought to possess various medicinal properties, i.e., for the treatment of minor burns, skin abrasions, and irritations (Ni et al. 2004; Hamman 2008). The gel has been used to treat a number of diseases e.g. gingivitis and against herpes simplex viruses (Tarro 1993; Krinsky et al. 2003). Second component, i.e., outer pericyclic tubules produce a reddish yellow exudate that has powerful laxative properties (Wynn 2005).

The polysaccharides present in the *Aloe* gel are not stable. They degrade under stress conditions such as heat, presence of acid and enzymatic activities (Turner et al. 2004). Therefore, standardization of the production process is required in order to preserve the natural biological activity of the aloe gel (Turner et al. 2004; Hamman 2008).

16.8 In Vitro Cultivation of *Aloe vera*

The medicinal plants are of utmost importance to pharmaceutical industries. They are mostly harvested from the wild, which causes serious problems like depletion of resources, extinction of rare species, seasonal collections, incorrect identification, and adulterations in dried materials. The possible solution for many of these issues is cultivation of medicinal plants under in vitro environment. Cultivation of *Aloe* has been adopted in many countries for commercial purposes. It is propagated vegetatively, where a single plant may produce two to three side shoots per year,

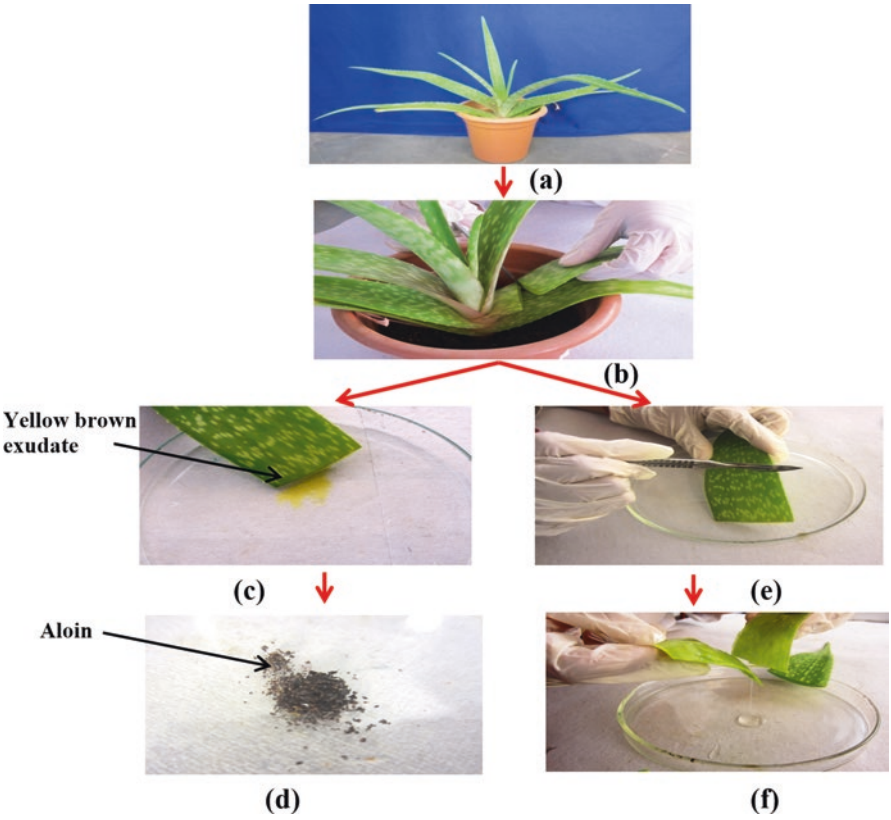

Fig. 16.5 The picture depicts the extraction of Aloe gel and the anthraquinone 'aloin' (**a**) Six month old *Aloe vera* plant (**b**) leaf detached with the help of scalpel. Aloe leaf contain two main constituents: (**c**) the yellow brown sap exudating from the cut portion, and (**d**) upon freeze drying forms a dark brown powder, called aloin, one of the secondary metabolite of *Aloe vera*. Secondly, (**e**, **f**) the Aloe gel, an important constituent of most of the cosmetic products and therapeutics. (Sharma et al. 2014b)

thus making availability of planting material in good quantity and quality, a problem.

Tissue culture technique has been widely used for propagation of plantlets. A variety of explants have been used for the micro propagation of *Aloe vera* plantlets such as, shoot tip, auxiliary bud, stem cuttings and rhizomes (Abrie and Staden 2001; Sharma et al. 2014a; Ahmed et al. 2007). Sucrose has been used for the bud initiation on semi-solid MS medium by Liao et al. (2004). MS medium supplemented with 2 mg/l BA +0.3 mg/l NAA as the best medium for micro- propagation of Chinese *Aloe vera*.

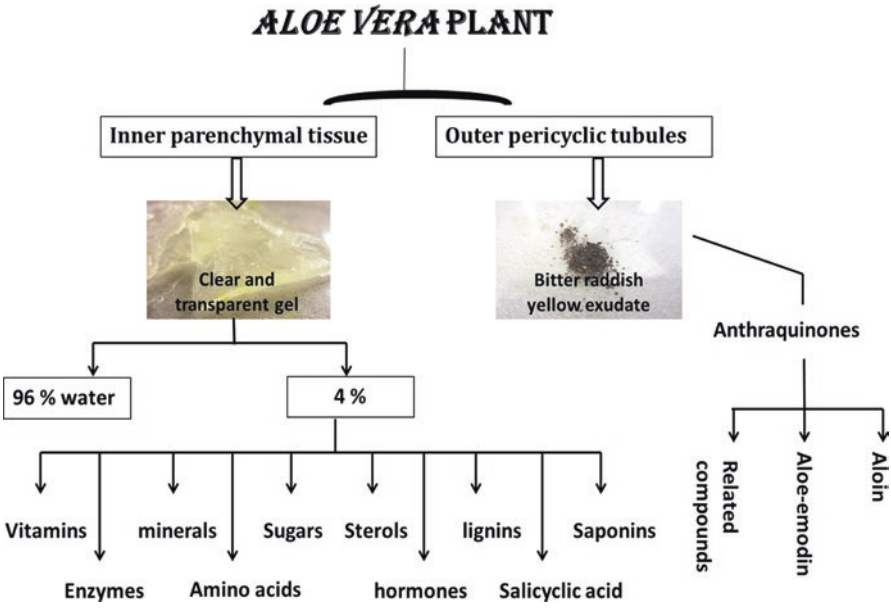

Fig. 16.6 Components of *Aloe vera* leaf. (Sharma et al. 2014b)

Sharma et al. (2014a) established the culture of *A. vera* using underground rhizome and reported the best response (85% shoot generation) on MS media containing 0.5 mg/l BAP + 0.5 mg/l NAA (9 ± 1 shoots per explants). The shoots at this concentration were green and healthy and shoot proliferation started very early and reached the maximum height (7 ± 0.5 cm) (Mean±SD) (Fig. 16.6). Maximum shoot multiplication was observed on MS medium having 0.5 mg/l BAP (10.67 ± 1.15 shoots per explants). MS medium containing 1.5 mg/l BAP gave 5.67 ± 0.57 shoots per explants. MS basal medium proved to be best for rooting (99.97%) (Sharma et al. 2014a) (Fig. 16.6). Abadi and Kaviani (2010) reported the best rooting on 0.5 mg/l BAP and 0.5 mg/l NAA containing MS media.

A. vera explants secrete phenolics that lead to browning and often death of the explants (Abrie and Staden 2001). Due to low propagation rate of Aloe, it is very difficult to meet its high industry demand (Natali et al. 1990; Abdi et al. 2013). Thus Micro propagation has gained importance for rapid production of plantlets (Pandhair et al. 2011; Roy and Sarkar 1991; Abrie and Staden 2001; Meyer and Staden 1991; Abadi and Kaviani 2010; Marfori and Malasa 2005; Corneanu et al. 1994). The micropropagated plantlets in a study performed better than conventionally propagated plantlets (Gantait et al. 2011). Roy and Sarkar (1991) used Poly vinyl Pyrollidone (PVP) to reduce the secretion of phenolics from the explants. Sharma et al. (2014a) however, avoided the phenolics by frequent transferring and trimming the ends of the plantlet until the cultures were established.

16.9 Strategy to Promote Growth

16.9.1 Symbiotic Interaction of *Piriformospora indica* with *Aloe vera*

Rooted plantlets of *A. vera* (4 weeks old) were co-cultivated with *P. indica* in solidified MS medium. *P. indica* was cultured on solidified potato dextrose medium in the dark at 28 ± 2 °C. Discs (8 mm) of *P. Indica* having spores and fully grown hyphae (1 week old culture) were placed 1 cm apart from explants. The non-inoculated plantlets acted as control. Cultures were incubated at 25 ± 2 °C, Relative humidity of 70% with 16 h light (2500 lx) provided by cool white fluorescent tubes, in Plant tissue culture laboratory. After 6 weeks of co-cultivation, plantlets from each set were taken and measured for various parameters, such as shoot height, root height, number of shoots, number of roots and fresh weight.

P. indica colonized *A. vera* plantlets had enhanced growth characteristics as compared to the non-colonized plantlets (Fig. 16.7). Shoot and root length of *P. indica* colonized plantlets increased by 81% and 64%, respectively. Fungus also enhanced the number of shoots (13%) and number of roots (93%) per plantlet. The increase in number of shoots was non-significant. Increase of 264% in fresh weight was

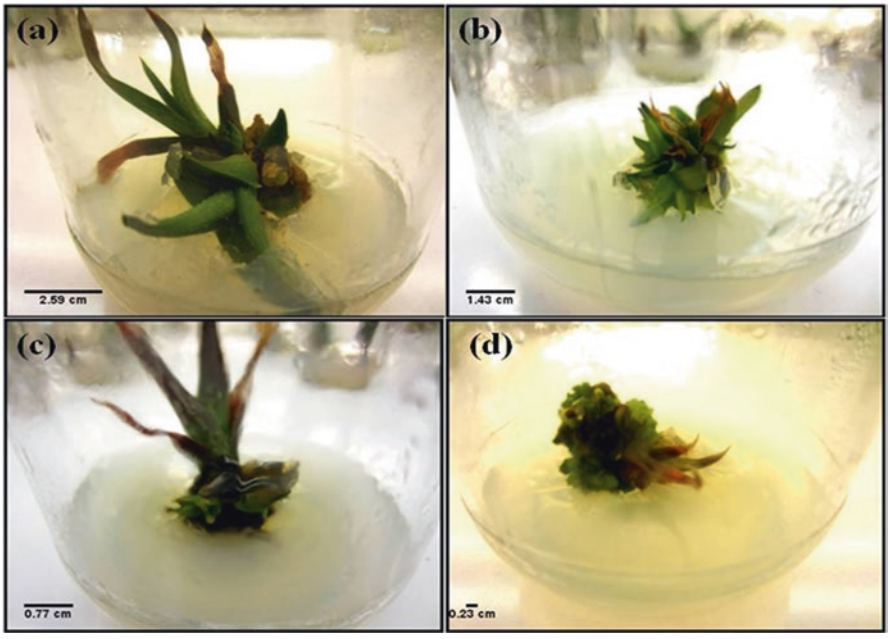

Fig. 16.7 Shoot proliferation on various combinations of hormones. Upon establishment of culture, 4 weeks old *A. vera* plantlets were grown at different concentrations (mg/l) of growth hormones (BAP and NAA) in MS medium. MS medium was supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar at a pH of 5.8. Cultures were incubated in a temperature controlled room at 25 ± 2 °C with 16 h light (2500 lux), provided by cool white fluorescent tubes. These photographs were taken at the end of 5 weeks **(a)** 0.5 BAP + 0.5 NAA **(b)** 0.5 BAP **(c)** 1.5 BAP **(d)** 2.5 BAP

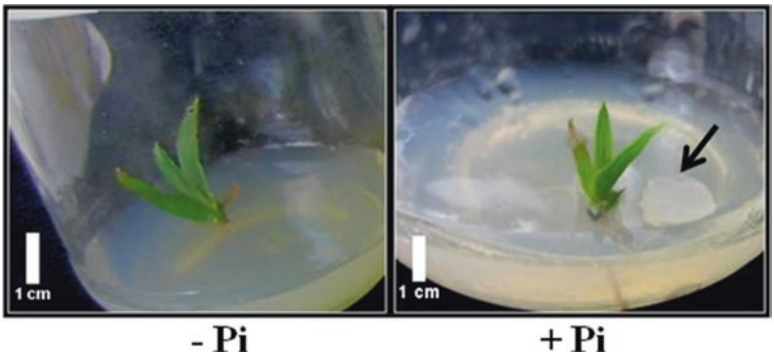

Fig. 16.8 Four weeks old rooted *Aloe vera* plantlets on 1st day of co-cultivation with *P. indica*. The arrow indicates the disc of 1 week old culture of *P. indica* having spores and actively growing hyphae. −Pi represents the *A. vera* plantlets without inoculation of *P. indica*, whereas +Pi represents the *A. vera* plantlets with inoculation of *P. indica*. Cultures were incubated in a temperature controlled room at 25 ± 2 °C with 16 h light (2500 lux), provided by cool white fluorescent tubes

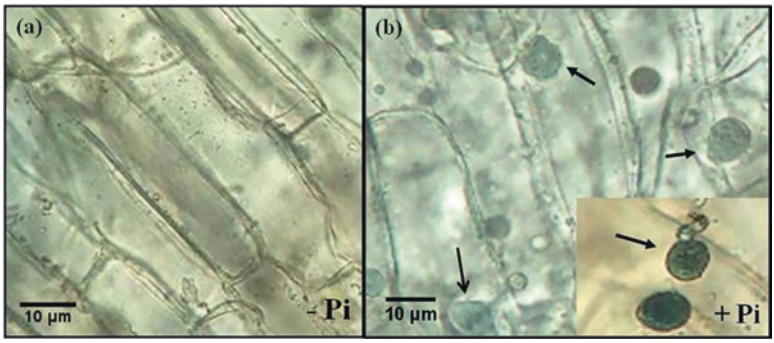

Fig. 16.9 Micrographs showing root cortical cells from 10 weeks old *Aloe vera* plantlets **(a)** control or non-colonized **(b)** after 6 weeks of co-cultivation with *P. indica*. The colonized roots (**b**) show the presence of spores of *P. indica* (indicated by arrows) at 40× magnification under bright field illumination. (Sharma et al. 2014a)

observed in *P. indica* colonized plantlets as compared with the non-colonized plantlets (Sharma et al. 2014a).

Root of *A. vera* plantlets were observed for colonization. Root segments (1 cm) from each set were examined under light microscope. The distribution of chlamydospores within the root cortex was taken as an index of colonization. Treated plantlets of *A. vera* had 68% colonization (Fig. 16.8b) in comparison to 0% of control (Fig. 16.8a).

The polysaccharides and anthraquinone derivatives present in Aloe gel have various therapeutic properties (Ammar et al. 2010). Aloin (yellow brown) is one of the major anthraquinone present in *Aloe* sap is used as a laxative, anti inflammatory and anti cancer agent (Lee et al. 2014).The aloin content as determined by HPLC was found to be 1.28 ± 0.057 mg aloin/g of leaf exudate for the *P. indica* colonized plantlets and 0.844 ± 0.01 for the non-colonized ones, marking an overall increase of 53% (Fig. 16.9) (Sharma et al. 2014a).

16.10 Effect of *P. indica* Inoculation, Salinity Treatment and Duration of Salt Exposure on Physiological and Biochemical Characteristics of *Aloe vera* Plantlets: A Case Study

Two months old in vitro grown *A. vera* plantlets were transferred to pots containing 1: 1: 1 mixture of soil, vermiculite and sterilized compost (v/v). They were divided into two sets: Uninoculated (Control) and inoculated with *P. indica*. The culture of fungus was maintained on Hill and Kaefer medium (Hill and Kaefer 2001) maintained at 28 ± 2 °C with continuous shaking at 120 rpm. The plantlets were inoculated by placing wet fungal mycelium (2 week old) with inoculum density of 2.5%, 2 cm below the surface of roots (Evelin and Kapoor 2014). Autoclaved inoculum was used for control plantlets. The plantlets were kept at relative humidity of 75% and temperature of 24 ± 1 °C with light intensity of 12,000 lx 16/8 h, day/night cycle.

Salinity stress severely affected the % root colonization of *P. indica*. The inoculated plantlets with no salt stress exhibited 80.1 ± 1.1% root colonization. However, the percentage root colonization significantly reduced (73.1 ± 1.3% and 67.1 ± 1.7%) for the inoculated plantlets at low or moderate salinity stress (100 mM and 200 mM). The percentage root colonization which were subjected to highest level of stress had 51.7 ± 2.2% root colonization. Non inoculated plantlets didn't show any colonization.

Inoculated plantlets of *A. vera* had better growth, biomass and survival as compared to non-inoculated plantlets (Fig. 16.10a–c). Various morphological parameters like shoot and root height, number of leaves, number of roots, leaf diameter, stem thickness and fresh weight were compared (Table 16.1).

A. vera plantlets inoculated with *P. indica* also had higher gel and aloin content (Sharma et al. 2014a, 2015, 2017b). The inoculated plantlets had better biochemical characteristics as compared to non-inoculated plantlets. Secondary metabolites like Phenolics, Flavonoids and Flavonols have been attributed to give medicinal properties to *A. vera* plantlets. The inoculated plantlets had significantly higher phenolics and flavonoids content in their leaves, which increased with increase in salinity stress (Fig. 16.12). It further increased when the plantlets were inoculated with *P. indica.* Inoculation with *P. indica* has no significant effect on root flavonoids content (Table 16.2). This increase in secondary metabolites could be due to elicitation of plant defence mechanism in response to endophyte colonization (Gao et al. 2010). The inoculated plantlets of *A. vera* had higher gel content. Salinity stress resulted in reduction in gel content of non-inoculated plantlets by 95% and inoculated by 80% after 2-month of salinity stress (Fig. 16.11) (Sharma et al. 2014a, b). Only few reports on the potential application of *P. indica* for enhancing secondary

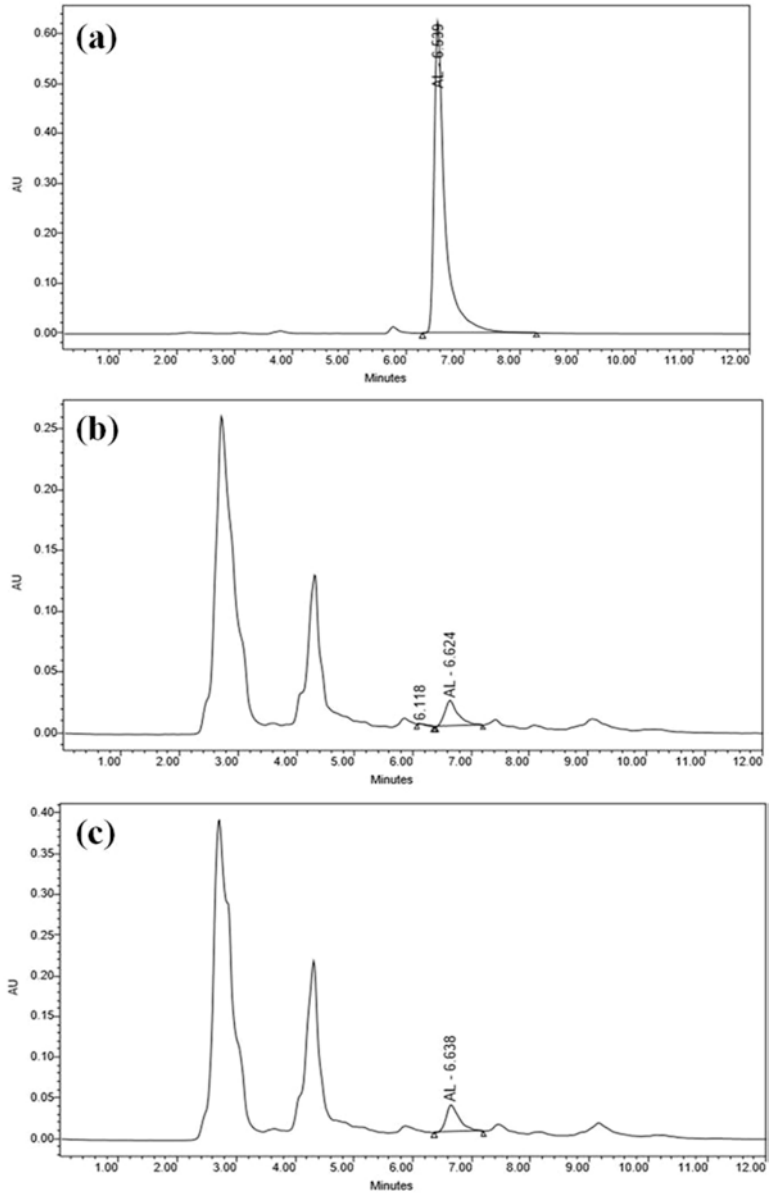

Fig. 16.10 Effect of *P. indica* on aloin content of 26 weeks old *Aloe vera* plantlets, after 16 weeks of acclimatization **(a)** standard aloin **(b)** control plants **(c)** plants treated with *P. indica*. (Sharma et al. 2014a)

Table 16.1 Effect of *P. indica* inoculation (−Pi and + Pi), salinity treatment (0, 100, 200 amd 300 mM NaCl) and duration of salinity exposure (1 MAT and 2 MAT) on growth parameters of *A. vera* plantlets

Growth parameters			NaCl concentration (mM)			
			0	100	200	300
Shoot length (cm)	1 MAT	−Pi	24.1 ± 3.1 b	22.2 ± 2.9 b	20.0 ± 3.6 b	17.9 ± 1.5 ab
		+Pi	32.2 ± 3.5 d	29.3 ± 2.7 e	22.8 ± 2.3 c	20.6 ± 2.3 ac
	2 MAT	−Pi	38.8 ± 2.1 a	34.6 ± 1.5 c	28.8 ± 1.8 e	24.5 ± 1.7 g
		+Pi	44.3 ± 2.1 b	38.7 ± 1.7 d	32.2 ± 2.3 f	28.8 ± 2.2 h
Root length (cm)	1 MAT	−Pi	12.0 ± 2.7 b	9.2 ± 0.8 a	7.9 ± 1.1 a	5.1 ± 1.0 f
		+Pi	14.8 ± 0.9 c	12.5 ± 1.3 d	9.7 ± 1.7 e	7.8 ± 1.6 g
	2 MAT	−Pi	14.8 ± 1.5 a	13.7 ± 1.0 a	11.8 ± 1.6 d	9.0 ± 1.4 e
		+Pi	17.9 ± 1.3 c	16.4 ± 0.8 b	15.3 ± 0.9 b	11.7 ± 1.7 f
Number of leaves	1 MAT	−Pi	7.4 ± 0.5 b	6.3 ± 0.8 a	5.6 ± 0.5 d	4.4 ± 0.5 e
		+Pi	8.4 ± 0.7 c	6.4 ± 0.5 a	6.6 ± 0.5 a	5.6 ± 0.6 f
	2 MAT	−Pi	12.3 ± 0.9 a	7.9 ± 0.9 b	7.6 ± 1.1 b	6.0 ± 0.9 c
		+Pi	12.9 ± 1.1 a	8.1 ± 1.1 b	7.9 ± 0.7 b	7.2 ± 0.8 b
Number of roots	1 MAT	−Pi	11.1 ± 2.3 b	10.1 ± 1.3b	8.1 ± 0.7 a	5.9 ± 1.0 d
		+Pi	12.8 ± 1.3 c	11.6 ± 1.0 c	9.3 ± 1.3 a	7.6 ± 0.8 e
	2 MAT	−Pi	13.4 ± 1.5 c	10.9 ± 1.5 e	8.0 ± 1.5 a	7.1 ± 1.1 a
		+Pi	15.2 ± 1.7 d	12.6 ± 1.5 f	9.6 ± 1.4 b	8.6 ± 1.2 b
Stem thickness (mm)	1 MAT	−Pi	17.8 ± 1.5 c	14.4 ± 1.0 a	13.2 ± 1.8 a	11.3 ± 1.3 b
		+Pi	20.2 ± 1.8 d	15.9 ± 1.4 e	14.1 ± 1.0 a	13.2 ± 1.4 a
	2 MAT	−Pi	23.6 ± 0.8 a	19.1 ± 1.0 c	16.1 ± 1.5 e	11.8 ± 1.8 g
		+Pi	27.5 ± 1.1 b	21.9 ± 1.7 d	18.7 ± 1.4 f	14.3 ± 1.9 h

Leaf diameter (mm)	1 MAT	−Pi	3.0 ± 0.4 a	3.0 ± 0.4 a	2.4 ± 0.4 b	2.0 ± 0.4 b
		+Pi	4.0 ± 0.7 c	3.8 ± 0.6 c	3.5 ± 0.7 c	2.9 ± 0.6 d
	2 MAT	−Pi	5.1 ± 0.7 b	4.3 ± 0.7 a	3.9 ± 0.5 a	2.5 ± 0.4 f
		+Pi	6.5 ± 0.6 c	5.7 ± 0.6 d	4.9 ± 0.3 e	3.4 ± 0.5 g
Fresh wt of whole plant (g)	1 MAT	−Pi	45.3 ± 5.2 a	42.1 ± 4.9 a	39.2 ± 5.4 a	31.9 ± 4.1 c
		+Pi	80.2 ± 1.1 b	76.5 ± 5.9 b	71.8 ± 4.1 b	66.8 ± 5.8 b
	2 MAT	−Pi	90.4 ± 2.9 a	84.3 ± 5.1 a	67.1 ± 5.0 b	34.7 ± 3.3 c
		+Pi	149.3 ± 5.9 g	130.2 ± 2.9 f	100.4 ± 14.9 e	69.7 ± 9.8 d
Fresh wt of leaf (g)	1 MAT	−Pi	37.7 ± 6.0 a	36.1 ± 5.9 a	33.2 ± 4.3 a	26.8 ± 5.5 b
		+Pi	71.0 ± 2.1 c	67.6 ± 4.9 c	61.9 ± 3.6 d	60.8 ± 3.7 d
	2 MAT	−Pi	80.1 ± 3.7 a	76.7 ± 5.3 a	60.1 ± 8.7 d	29.1 ± 2.6 f
		+Pi	135.9 ± 7.1 b	117.7 ± 7.2 c	89.2 ± 17.8 e	61.3 ± 12.3 g
Fresh wt of root (g)	1 MAT	−Pi	7.3 ± 1.9 c	6.9 ± 0.5 c	6.8 ± 1.6 ac	5.9 ± 0.8 bc
		+Pi	9.6 ± 1.5 a	8.4 ± 1.7 a	8.1 ± 1.6 a	6.5 ± 1.2 b
	2 MAT	−Pi	11.1 ± 1.5 a	10.4 ± 1.0 a	8.6 ± 0.8 c	6.4 ± 1.2 e
		+Pi	14.1 ± 3.2 b	13 ± 2.2 b	11.1 ± 1.8 d	9.1 ± 0.9 f

The plantlets were 5-month old after stress exposure of 2-month. Results have been expressed as Mean ± SD. Different letters indicate the values were significant at $P < 0.05$ as determined by Analysis of Variance (ANOVA). Each data set represents an average of 14 replicates. −Pi: non- inoculated; +Pi: inoculated; MAT: Months after salinity treatment (Sharma et al. 2017a)

Table 16.2 Effect of *P. indica* inoculation (−Pi and +Pi), salinity treatment (0, 100, 200 amd 300 mM NaCl), duration of salinity exposure (1 MAT and 2 MAT) and tissue (leaf and root) on biochemical parameters of *A. vera* plantlets

Biochemical parameters				NaCl concentration (mM)			
				0	100	200	300
Total Phenol (mg gallic acid equivalent/g dry wt)	Leaf	1 MAT	−Pi	2.4 ± 0.2 a	3.2 ± 0.4 a	3.5 ± 0.3 a	5.1 ± 0.4 c
			+Pi	6.0 ± 0.5 b	6.5 ± 0.7 b	7.1 ± 0.8 b	8.1 ± 0.5 d
		2 MAT	−Pi	5.5 ± 0.2 d	7.3 ± 0.6 a	8.2 ± 0.7 a	10.7 ± 0.6 c
			+Pi	10.7 ± 0.9 c	12.1 ± 0.9 f	13.5 ± 1.2 b	14.5 ± 0.9 b
	Root	1 MAT	−Pi	1.2 ± 0.2 a	1.4 ± 0.2 ab	1.6 ± 0.1 ab	2.0 ± 0.3 b
			+Pi	1.9 ± 0.6 c	2.3 ± 0.6 cd	2.5 ± 0.7 cd	2.8 ± 0.7 cd
		2 MAT	−Pi	2.0 ± 0.4 a	2.5 ± 0.7 a	2.9 ± 0.7 a	3.3 ± 0.5 a
			+Pi	2.8 ± 0.3 b	3.2 ± 0.5 b	3.8 ± 0.6 b	4.0 ± 0.4 a
Total Flavonoids (mg quercetin equivalent/g dry wt	Leaf	1 MAT	−Pi	1.2 ± 0.1 ac	1.4 ± 0.1 bc	1.4 ± 0.1 dc	1.9 ± 0.3 f
			+Pi	1.3 ± 0.2 ah	1.4 ± 0.2 bh	1.7 ± 0.2 e	2.5 ± 0.3 g
		2 MAT	−Pi	3.3 ± 0.2 a	3.9 ± 0.2 bh	4.4 ± 0.2 d	3.9 ± 0.3 fh
			+Pi	3.5 ± 0.2 a	5.7 ± 0.2 ci	6.6 ± 0.2 e	5.5 ± 0.1 gi
	Root	1 MAT	−Pi	0.6 ± 0.1 a	1.2 ± 0.1 bc	1.4 ± 0.1 bc	1.8 ± 1.7 d
			+Pi	0.7 ± 0.1 a	1.0 ± 0.2 bd	1.3 ± 0.1 bd	1.9 ± 0.2 d
		2 MAT	−Pi	2.4 ± 0.2 ae	2.7 ± 0.1 be	3.5 ± 0.2 c	4.5 ± 0.2 d
			+Pi	2.6 ± 0.2 af	2.9 ± 0.4 bf	3.7 ± 0.2 c	4.4 ± 0.2 d

Total Flavonols (mg quercetin equivalent/g dry wt)	Leaf	1 MAT	−Pi	2.6 ± 0.2 b	4.3 ± 0.1 ah	4.6 ± 0.4 dh	3.9 ± 0.2 f
			+Pi	3.8 ± 0.1 c	4.4 ± 0.2 ai	5.4 ± 0.1 e	4.5 ± 0.1 gi
		2 MAT	−Pi	4.3 ± 0.1 a	4.8 ± 0.1 a	6.4 ± 0.1 d	4.9 ± 1.0 a
			+Pi	5.5 ± 0.2 c	7.2 ± 0.1 b	9.3 ± 1.3 e	7.0 ± 0.5 b
	Root	1 MAT	−Pi	1.3 ± 0.1 f	2.7 ± 0.1 e	2.8 ± 0.1 ae	3.3 ± 0.1 b
			+Pi	1.8 ± 0.1 c	2.4 ± 0.1 d	2.9 ± 0.1 a	3.4 ± 0.1 b
		2 MAT	−Pi	2.7 ± 0.1 f	3.6 ± 0.1 a	4.2 ± 0.1 b	4.5 ± 0.1 c
			+Pi	3.5 ± 0.2 d	3.7 ± 0.2 ad	4.4 ± 0.2 be	4.3 ± 0.5 ce
Total Soluble Sugar (mg glucose equivalent/g dry wt)	Leaf	1 MAT	−Pi	22.5 ± 3.0 ae	25.1 ± 2.8 bef	27.8 ± 2.5 caf	25.4 ± 3.6 def
			+Pi	26.0 ± 3.1 ag	29.6 ± 4.4 gb	30.2 ± 5.5 cg	26.1 ± 3.3 dg
		2 MAT	−Pi	24.7 ± 3.8 a	23.8 ± 3.6 ba	22.9 ± 2.8 bca	19.7 ± 3.5 bd
			+Pi	25.5 ± 3.1 ae	24.7 ± 1.6 bed	23.5 ± 3.7 ced	20.8 ± 6.0 d
Reducing Sugar (nmol glucose equivalent/g dry wt)	Leaf	1 MAT	−Pi	61.5 ± 8.1 a	116.3 ± 4.0 c	175.1 ± 10.4 e	117.4 ± 5.6 c
			+Pi	131.4 ± 11.8 b	164.4 ± 20.2 d	196.8 ± 12.3 f	142.5 ± 31.6 bd
		2 MAT	−Pi	546.8 ± 28.3 a	430.9 ± 43.9 c	234.2 ± 26.8 d	176.4 ± 22.0 d
			+Pi	180.8 ± 17.9 b	481.1 ± 58.4 c	364 ± 57.8 e	272.7 ± 58.5 g

The plantlets were 5-month old after stress exposure of 2-month. Results have been expressed as Mean ± SD. Different letters indicate the values were significant at $P < 0.05$ as determined by ANOVA. Each data set represents an average of 6 replicates (total solublesugar and reducing sugar: leaf tissue only, 8 replicates). −Pi: non- inoculated; +Pi: inoculated; MAT: Months after salinity treatment (Sharma et al. 2017a)

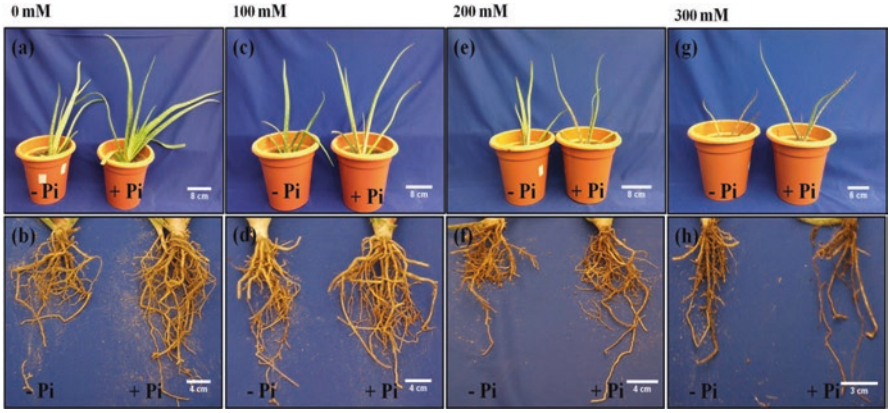

Fig. 16.11 Plant growth phenotype of five months old *P. indica* inoculated (2.5% inoculum density) and non-inoculated *Aloe vera* plantlets treated with different concentrations of NaCl, ranging from 0, 100, 200 and 300 mM. Photographs were taken after two months of salt treatment under biohardening facility. (Sharma et al. 2017a)

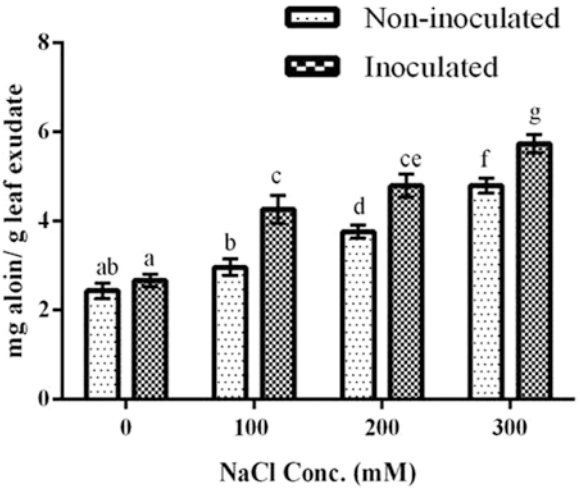

Fig. 16.12 Effect of *P. indica* inoculation (inoculated and non-inocualted) and salinity treatment (0, 100, 200 and 300 mM) on Aloin content of *A. vera*. Each data set represents an average of 3 replicates. The error bars represent SE. Different letters on the bar indicate that the values differ significantly at $P < 0.05$ as determined by Analysis of Variance. (Sharma et al. 2017a)

metabolite content of plants (Satheesan et al. 2012; Bajaj et al. 2014; Sharma et al. 2014a, 2015; Kilam et al. 2015) under salinity stress are available.

16.11 Conclusion and Future Perspectives

A vast majority of temperate and tropical plant species grow in the field under the influence of mutualistic association with fungi. A number of microbes have shown to improve the growth of their hosts after root colonization, the list includes,

Phialocephala fortinii, *Cryptosporiopsis* spp., dark septate endophyte, *Piriformospora indica*, *Fusarium* spp. and *Cladorrhinum foecundissimum* to name a few (Schulz 2006). These fungi colonize the roots and rhizosphere and branch more frequently and extensively in comparison to the plant roots, hence forming a ramified network which increases the absorptive capacity of roots and allows the plant to have a better access to greater quantity of water and minerals required for nutrition (Clark and Zeto 2000; Schulz 2006).

This synergism increases the nutrient availability and improves the rhizospheric and soil conditions, resulting in better growth and improved vigour of the plant. Also, it increases the availability of carbon source to the fungus in the form of sugars, amino acids and vitamins which are essential for its growth (Clark and Zeto 2000). This ultimately results in the dilution of toxic ion effect. Most of the studies based on beneficial symbiotic association of plant and microbes have focussed on rhizobia and Arbuscular Mycorrhizal (AM) Fungi. *Piriformospora indica* is an easily cultivable fungus (Sebacinacae) that colonizes the roots of enormous plant species, in a manner similar to AM fungi. It provides several benefits to the host, other than merely plant growth promotion, such as acting as an important medicinal ingredients, economical benefits etc. It acts as biofertilizer, bioprotector, immunoregulator etc. It is a very important tool for biological hardening of tissue culture raised plants (Bagde et al. 2010; Varma et al. 2013). It induces tolerance against salt stress and resistance against various roots and shoots pathogens (Waller et al. 2005; Serfling et al. 2007). Thus, it proves to be a wonderful model organism for the investigation studies of mutualistic plant microbe interactions. It further enables the identification of compounds, which may enhance the growth and productivity of useful plants (Peškan-Berghöfer et al. 2004). The study conducted by Sharma et al. (2014a, 2017a) aims at interaction between the *Aloe vera* L. with endosymbiotic fungus *P. indica*. The beneficial symbiotic interaction of plant and fungus provides a promising tool for the better establishment of in vitro grown plants and for enhancing various phyto-chemicals and secondary metabolites present in them. These secondary metabolites not only protect the plant from various oxidative stresses, but also the animals who feed on it. Thus, *P. indica* was cultivated under in vitro environment and applied as inocula to micro propagated *A. vera* plantlets under controlled experimental conditions so that its potential on morphogenesis and secondary metabolites of the test plant could be analyzed.

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Nanotechnological Interventions for Improving Plant Health and Productivity

17

Anjali Pande and Sandeep Arora

Abstract

Green revolution was responsible for ushering in a significant enhancement in crop productivity. It turned agriculture in India from a household occupation to an industrial system. This phase of rapid industrialization was brought about by the use of improved crop varieties, modern methods of cultivation and use of pesticides and herbicides. However, uncontrolled and excessive use of synthetic growth enhancers, pesticides and fungicide chemicals, has given rise to problems related with soil health, sustainability of productivity, environment stability and health etc. As a result, novel alternative approaches utilizing environmentally benign bio-fertilizers/bio-pesticides, as substitutes to harmful agro-chemicals, came into use so as to ensure human health and environment safety. In more recent times, population explosion and climate changes due to global warming have put additional strain on agricultural scientists to grow sufficient food from less arable land. Therefore, the present era focuses on innovative technologies which are oriented towards addressing the current challenges of sustainability, food safety and security and environment health. Researchers all over the world are exploring the feasibility of various innovative approaches, including nano-technological interventions, for improvement in agricultural sector. State-of-art research has identified the potential of highly innovative nano-material applications in food production. Nanotechnology, though not a recent concept, offers a diverse array of conceptual applications in the field of agriculture aimed at improving plant health and productivity. Nano-based products like nano-fertilizers, nano-diagnostics, nano-fertigation products and nano-pesticides, etc. offer multifaceted advantage over their macro-chemical counterparts. Nano-fertilizers and fertigation based products intend to optimize nutrient use by crops

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through exploiting properties unique to nanoparticles. Their application is more effective and at lower concentrations than the huge amounts of chemical fertilizers which are responsible for soil degradation and contribute to pollution of surface and underground water resources. The use of smart delivery system enables nutrient management with minimal nutrient losses and maximal yield optimization. Additionally, nano-based smart sensors are useful in precision farming thus enabling maximum productivity from crops while reducing the input costs incurred on excessive nitrogen, phosphorus and potassium fertilizers, irrigation, etc., through rigorous monitoring of physical growth variables and regulated release mechanisms. Moreover, nano-sensors and nano-biosensors are useful for monitoring of soil pH, and soil composition parameters. Other interesting areas of scientific investigations are genetic manipulation of plants through nano-based gene delivery systems and enhancing the nutraceutical value of crops through nano-biofortification. Despite the huge amount of patents and published data on the numerous benefits of nanotechnology in farming sector, this technology has not yet been exploited to its fullest potential. However, it is apparent that nanotechnology marks a new horizon and is a promising technology for revolutionizing modern agriculture.

Keywords
Nanofertilzers · Nano-bio-fortification · Nano-biotechnology · Plant stress · Precision farming · Sustainable agriculture

17.1 Introduction

Nanoscience and nanotechnology involves the study, design, characterization, production, manipulation and application of materials at nanometer scale. Nanoscience and nanotechnology allows manipulation of materials at atomic and molecular levels, wherein the properties (physical, chemical and biological) differ significantly from those at the bulk (macro) scale. Nanotechnology combines knowledge from different disciplines: chemistry, physics and biology and is hence a truly multidisciplinary field (Brune et al. 2006) and hence can be applied across all the science fields. With the advent of twenty-first century 'nanoscience' has emerged as one of the most fascinating branches of modern science, which has revolutionized the field of material sciences. In the recent past innovations in nanoscience and nanotechnology have surfaced rapidly and brought forth novel applications in various spheres of human endeavor, including but not limited to diagnostics, computing, aviation, energy storage and production.

Technical innovations in the field of agriculture are specifically important with regard to addressing global challenges such as food and nutritional security, climate change and the limited availability of important plant nutrients such as phosphorus and potassium. Under such a scenario, nano-technological interventions in the field of agriculture play a vital role in sustaining food and nutritional security for the

masses. Although, the research on agricultural applications of nanomaterials is ongoing for largely a decade (Berger 2016), yet the real benefit of nanoscience in food production and allied sectors has not been realized to its fullest and commercialization of nano-based products is still in its infancy. Scientists are now trying to converge their knowledge of material sciences at the nano-scale, towards solving the recalcitrant problems facing world agriculture and also related environmental challenges, *viz* increasing productivity from the current genotypes, environmentally benign crop protection systems, protection of soil health etc., through intelligent applications of nanomaterials. This chapter introduces fundamental concepts about the prospective avenues of nanoscience and technology in developing sustainable agricultural and environment friendly practices. The current chapter encompasses the establishment, development and application of nanotechnology in agriculture with major focus on plant health and productivity.

17.1.1 Nanoscience and Nanotechnology

While scientists define nanotechnology in various ways, the United States Environmental Protection Agency (USEPA) defines it as "research and technology development at the atomic, molecular, or macromolecular levels using a length scale of approximately one to one hundred nm in any dimension; the creation and use of structures, devices and systems that have novel properties and functions because of their small size; and the ability to control or manipulate matter on an atomic scale" (USEPA 2007). With the rapid developments in nanoscience, the knowledge acquired and applied practically has lead to the design, characterization, production and application of structures, devices and systems by controlling shape and size at nano meter scale. This has given rise to what is being referred today as nanotechnology.

Nanomaterials are usually classified into three groups, on the basis of the number of dimensions which lie within the nanometer range. These include (i) one dimension nanomaterials (e.g. thin films or monolayers and multilayered materials), (ii) two dimension nanomaterials (e.g. carbon nanotubes, nano wires and nano-rods) and (iii) three dimensional nanomaterials (e.g. dendrimers, Quantum Dots and Fullerenes (C_{60}) (Hett 2004).

The nanomaterials used for research, specifically in agricultural research are normally nano-sized materials that exhibit properties which are different from those obtained from the same chemical composition but with larger (macro) particle size. The commonly used nanotechnology-based novel agro-products include nano-sensors, nano-fertilizers, nano-pesticides and nano-formulations for plant productivity. A vast array of nanomaterials have been experimented, for helping to reduce the consumption of deleterious agrochemicals, for increasing the availability of micro and macro nutrients etc. These developments also include, but are not limited to, the use of smart nano-delivery systems which are useful in optimizing essential nutrient delivery and targeted release of pesticides. Such practices have been reported to produce higher yield from the current

genotypes in a sustainable manner. Nanotechnology has also entered the field of biotechnology in a big way and is currently a subject of intense investigation. Additionally, nanotechnology has also been used for development of agri based nano-composites and value addition to the agricultural produce for the benefit of farmers and other stake holders (Dwivedi et al. 2016).

17.1.2 Nanotechnology in Agriculture

Agriculture is the largest contributor to national GDP in various countries, as it provides raw materials for food and feed industries. A large proportion of global population has to tackle the problem of food insecurity, brought about by natural calamities like extreme droughts and flash floods (Joseph and Morrison 2006). It is a harsh truth that the till today, agricultural productivity is vastly reduced because of various biotic as well as abiotic factors. Under such circumstances, it becomes imperative to enhance the agricultural productivity with the limited resources (land, water, soil, etc.) available so as to feed the growing population. Novel and innovative approaches are the need of the hour for ensuring global food and nutritional security and to face the onslaught of climate change challenges.

Further, agricultural production and efficiency are highly dependent on the use of agrochemicals such as pesticides, fertilizers, etc. However, there are potential issues concerning water contamination and food products obtained from such treated lands that may pose serious threat to human health and ecosystem. Hence, precise and controlled input of such agrochemicals must be managed in order to reduce the risk (Kah 2015).

Agriculture has benefited from several important technological improvisations, including the use of hybrid varieties, and use of synthetic fertilizers, that had ushered in the era of green revolution. With the introduction of novel concepts of gene manipulation, agriculture moved from green to gene revolution, and now scientist are on the verge of a nano-revolution as an avenue of agricultural improvement (Parisi et al. 2015). The nano-revolution can change the entire concept of the current agricultural technology (Fig. 17.1). Tremendous opportunities and possibilities of nanotechnology can be exploited in agricultural through this nano-revolution. Some of these properties include high reactivity, enhanced bioavailability and bioactivity, adherence effects and surface effects of nanoparticles (Gutierrez et al. 2011). Moreover, the emergence of nanotechnology and the development of new nano-devices and nano-materials open up potential novel applications in agriculture. The nanomaterials would release pesticides or fertilizers at a specific time and targeted location. Nanoparticles tagged to agrochemicals or other substances and treated in a dose dependent manner could reduce the damage to other plant tissues and the amount of chemicals released into the environment (Nayan et al. 2016). The introduction of nano-based technologies in agriculture can revolutionize the current agricultural practices and well reduce the deleterious effects of current practices on the environment (Sekhon 2014; Liu and Lal 2015). A detailed account of nanotechnology based research activities in the agricultural sector are discussed in the following sections.

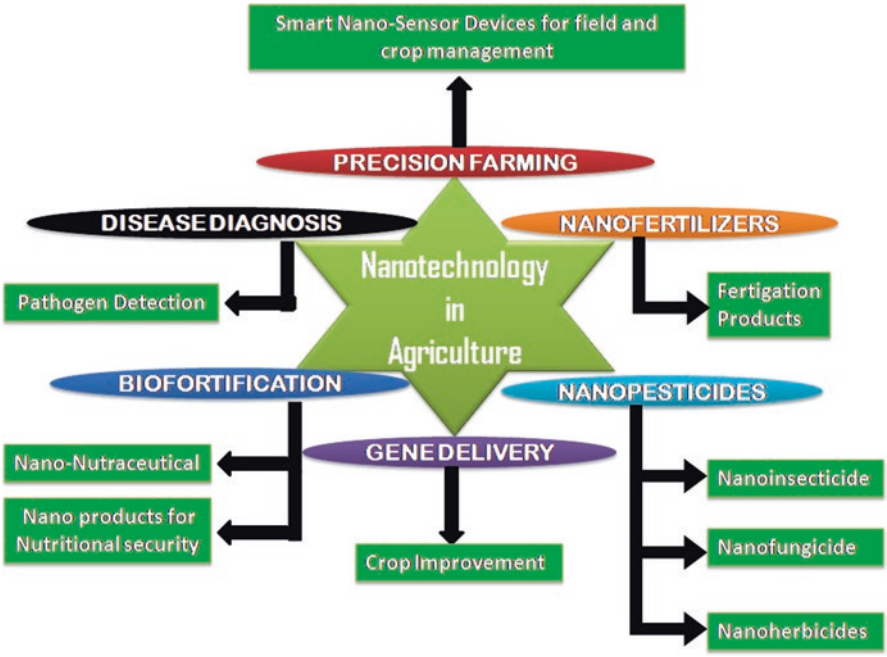

Fig. 17.1 Applications of nanotechnology in agriculture

17.1.3 Nano-based Products for Plant Health and Crop Improvement

The future of sustainable agriculture relies on technological innovations like nanotechnology which has the potential to ensure food and nutritional security for the burgeoning world population, without compromising environmental health. Nano based agricultural practices could lead to the production of sufficient nutritious food and decrease the negative impacts on the environment, induced by chemical fertilization. Furthermore, a judicious mix of biotechnology and nanotechnology could benefit agricultural systems and provide solutions for the recalcitrant problems facing the world agriculture. These innovative and potential applications include the development and use of smart delivery systems for fertilizers as well as other agro-chemicals, bio-enzymes in combination with bio-fertilizers (Calabi-Floody et al. 2018). Scope of nano-biotechnology in the field of agriculture is enormous. Here we shall discuss some important nanotechnology based products, developed for plant health and productivity, although a lot more products are currently at R&D stage.

17.1.3.1 Nano-fertilizers and Nano-fertigation Products

Agricultural production is largely dependent on fertilizers, as more than 50% of agricultural productivity is fertilizer dependent (Tabrizi et al. 2009). Synthetic

fertilizers like urea and other related NKP compounds play a pivotal role in improving the productivity across a broad spectrum of crops and have increased the food production considerably during the green revolution era. However, nutrient use efficiencies of conventional fertilizers hardly exceeds 30–35%, 18–20% and 35–40% for N, P and K respectively, and has largely remained constant for the past several decades. It is estimated that about 40–70% of nitrogen, 80–90% of phosphorus and 50–70% of potassium from the applied fertilizers is lost to the environment and can't be absorbed by plants, causing not only economic losses but environmental pollution as well (Ombodi and Saigusa 2000). Additionally, use of fertilizers even at higher doses does not guarantee improved crop yield, rather it may have harmful effects on the beneficial soil microflora, leading to soil degradation and contributes to pollution of surface and underground water resources (Qureshi et al. 2018). On the other hand nano-fertilizers intend to improve the nutrient use efficiencies in various crops, by exploiting the unique physico-chemical properties of nanomaterials (Subramanian et al. 2015). Nano-fertilizers include nano-capsules, nanoparticles and viral capsids for improvement in nutrient absorption and utilization by plants and also ensuring temporal and spatial specificity.

Synthesis of nano-fertilizers encompasses the fortification of different nutrients, individually or in combinations, onto specific carrier molecules having nano-dimensions. Several physical, chemical and biological methods are available to produce nano-based fertilizers as well as delivery materials and the targeted nutrients are loaded as cationic species (NH_4^+, K^+, Ca_2^+, Mg_2^+) and also anionic species after surface modification (NO_3^-, PO_4^{2-}, SO_4^{2-}) (Subramanian and Thirunavukkarasu 2017). Nano based fertilizers are designed to release the nutrients in a slow and steady manner over a long period of time which improves the nutrient use efficiency and loss of nutrient is substantially reduced thus taking care of environmental safety. The work on precision nano-fertilizer system is still at the developmental stage, but the detailed assessment reports clearly indicate that these customized fertilizers have a pivotal role to play in sustaining agricultural growth and productivity (Subramanian et al. 2015).

Recent research on cellular interactions of nano-fertilizers has yielded insights into the mode of action of these nano based fertilizers, underlining their positive effect on plant health and productivity through optimization of cellular metabolic pathways (Fig. 17.2). Examples of nano based fertilizer systems include ZnO nanoparticles as active ingredients in fertilizers (Milani et al. 2012) and hydroxyl-apathite urea-coated particles as additives in fertilizers for controlled release (Kottegoda et al. 2011). Scientists have also designed and explored the application of ZnO nanoparticles for coated fertilizer delivery (Milani et al. 2012).

Commonly used zinc fertilizers such as zinc oxides (ZnO) and zinc sulphates ($ZnSO_4 \cdot H_2O$) or ($ZnSO_4 \cdot 7H_2O$) cannot overcome zinc deficiency in plants (Mortvedt 1992). This limitation is caused because of non-availability of zinc in a biocompatible form. Specific derivatives like zinc oxide nanoparticles override this problem by providing a soluble and more amenable form of zinc to plants, and also because of greater reactivity of nano-based compounds in comparison to bulk zinc particles. As diffusion of dissolved zinc has been reported to be the principal route

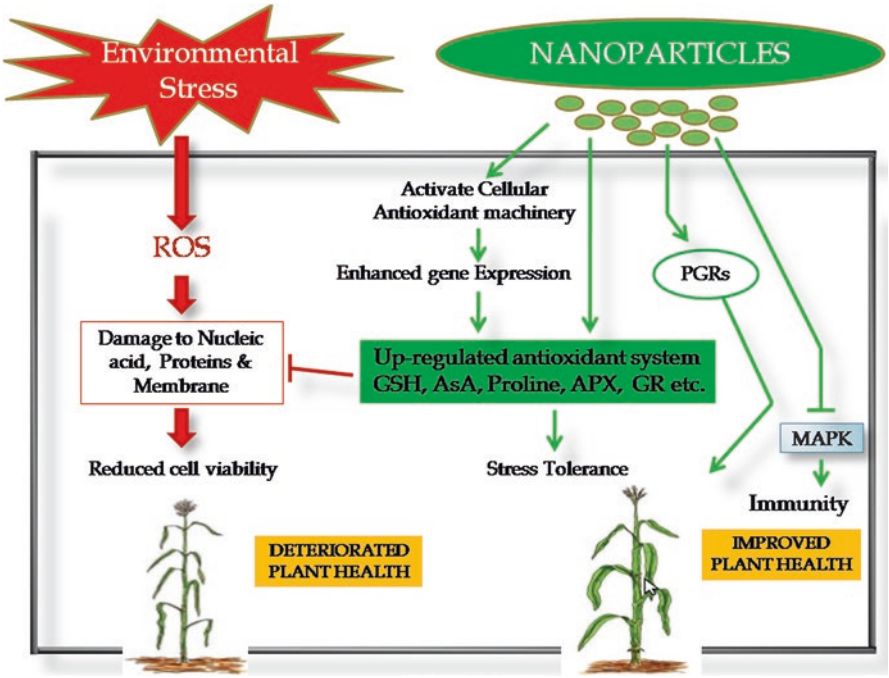

Fig. 17.2 Nanoparticle mediated cellular optimizations for improving plant health and productivity

for the translocation of zinc from soil to the plant roots following the dissolution process, therefore nano-zinc provides greater benefits in optimum quantities (Gangloff et al. 2006).

Researchers have also studied the effect of Zinc Sulfide nanoparticles (Nayan et al. 2016) and Iron Sulfide nanoparticles (Rawat et al. 2017) on the growth and yield of *Brassica juncea* under controlled as well as field conditions. The results obtained from the study indicate that these nanoparticles can potentially improve the redox status of the cell and can be employed for growth and yield enhancement. The study on iron sulfide nanoparticle (Rawat et al. 2017) suggests that the enhancement may be mediated through activation of carbon and nitrogen assimilatory pathways at specific growth stage.

A recent study by Badran and Savin (2018) suggests that the use of nano-fertilizer nano-urea modified with hydroxyapatite nanoparticles works as a source of N, P and Ca elements in bitter almond under saline conditions. These experiments indicate that pre-treatment of seeds with nano-fertilizers, under saline conditions, increases the germination percentage, stem length and diameter, root length and number of secondary roots per plant, as compared to the plants grown under similar conditions but provided conventional urea and ammonium sulfate fertilizers. Additionally, the fertilizer release was observed to be slow and continuous, resulting in stronger seedlings.

Use of Nano-fertigation products is another interesting innovation in the field of agricultural nanotechnology. Fertigation is basically a crop management practice that allows an adequate supply of fertilizer coupled with its timely application. Nano fertigation products are new-age agro-products used for specific and time dependent release of nutrients. Their use in agriculture increases crop yield and also improves nutrient use efficiency, leading to lesser wastage of fertilizers and controlled cost of cultivation. Nano-fertigation products are highly effective for accurate nutrient management in precision agriculture and match the crop growth stage according to soil and environmental conditions. Encapsulation of Nano-products accounts for slow and time dependent release of nutrients and increased surface area for nutrient adsorption. These lead to increased rate of photosynthesis for production of more dry matter and crop yield. It is may also help in lowering the impact of different biotic and abiotic stresses.

17.1.3.2 Nano-pesticides

Environmental vagaries, like abiotic and biotic stresses, continue to be the major limitations for agricultural productivity. It is well known that pests (insects, fungi and weeds) are predominantly present in the agricultural fields and are a threat to the agricultural productivity. Of these, insect pests and field weeds account for significant reduction in crop yields. Current estimates suggests that pests cause up to 25% loss in rice, 30% in pulses, 35% in oilseed crops, 20% in sugar cane and 50% in cotton (Dhaliwal et al. 2010). In order to enhance yield and productivity farmers use considerable amount of pesticides. Pesticides use has dramatic consequences both in developed and developing countries. The use of high amount of pesticides used to kill pests poses threat not only to the host crop or its products but also to the environment. Pesticides can contaminate soil, water, turf and other vegetation. In addition to killing insects or weeds, pesticides can be toxic to a host of other organisms including birds, fish, beneficial insects and non-target plants (Aktar et al. 2009). Pesticide chemicals may induce the generation of reactive oxidative species (ROS) leading to oxidative stress, resulting in crop productivity losses through alteration of the redox homeostasis (El-Shenawy et al. 2011). Furthermore, synthetic or fumigant pesticides used for plant protection and pest control in stores usually brings about resistance in these pests (Zamani et al. 2011). Therefore, the goal of sustainable agriculture is long term maintenance of natural resources and agricultural productivity with minimal adverse effects on the environment (Densilin et al. 2011).

For enhancing crop productivity the potential use of nanotechnology may include the development of nano-delivery systems for pesticides, nano-sized encapsulated pesticides with controlled and/or targeted release, as well as the stabilization of conventional pesticides with nanomaterials (Khot et al. 2012). It has been demonstrated that nano encapsulated pesticide formulations have slow active ingredient releasing properties with consequent improved solubility, higher specificity as well as stability in soil (Bhattacharyya et al. 2016). The enlisted desired properties are obtained through ‘encapsulation’, for protecting the active ingredients from premature degradation and/or increasing their presence in soil for a lager duration of time

(Prasad et al. 2017). The major advantage of the formulation of nano encapsulated pesticides is that the dosage of pesticides is highly reduced, hence lowering the exposure to human beings and the environment (Nuruzzaman et al. 2016). Thus, nanotechnology has the potential to develop less toxic and promising pesticide delivery systems for ensuring global food security without compromising the environmental health (Grillo et al. 2016).

17.1.3.3 Nano-insecticides

Long term and indiscriminate use of chemical insecticides in fields has resulted in destruction of soil micro-biota, degradation of soil texture and development of various disorders in human beings (Biyela et al. 2004). Due to these deleterious effects, scientists started discovering alternate strategies for controlling insect pests with minimum risks and zero side effects. Use of biological control agents against insect pests has shown several advantages over chemical pesticides. For instance, once introduced they have long term sustainability and usually do not interact with non-target pests. Additionally, there is no fear of environmental pollution as compared to chemical pesticides and it leaves no residues on consumable parts of plants. Integrated biological control of pest population is a useful and important management strategy. Many biological agents have been used for the bio-control of insect pests, but only bacteria and fungi are most important. The best example of bacteria and fungi used as biological control agents are *Bacillus thuringiensis* (Frederick and Caesar 2000) and *Beauveria bassiana* (Thungrabeab and Tongma 2007) respectively. However, there are several limitations associated with the application of biopesticides too such as high selectivity or host specificity, correct time of application, delayed effect or mortality, difficulty of culturing in large quantities and requirement of additional control measures (Pavela 2014).

Recent advancements in the field of nanoscience suggest that alternate methods of pest control in agricultural fields can be developed, by exploiting the improved surface area to volume ratios and higher reactivity of nanomaterials. These may potentially help to develop insecticides with improved efficacies and lower input costs. Although so far, only limited studies have been conducted on the development of alternate insecticides, yet the potential of new age nano-based insecticides indicate that they can even replace the prevalent genetic engineering strategies for developing insect resistance in important field crops like cotton.

The projection of new generation nano-insecticides as an alternative to genetically engineered crops has evoked a positive response in the society, as GE crops are not well accepted in various parts of the world, especially when used in edible crops. Nano-insecticides use specific nano engineered structures and have lower mass of active ingredient residues with a consequent smaller carbon foot-print (Bergeson 2010). Many research groups have studied the bio-efficacy of chemical and biological nanomaterials against insect pests. For instance, Chakravarthy et al. (2012), used inorganic nanoparticles of CdS, Ag and TiO_2 against *Spodoptera litura* under controlled laboratory conditions. Studies conducted by Rouhani et al. (2012) indicate the excellent bio-efficacy of silver and zinc nanoparticles against *Aphis nerii*. Yasur and Rani (2015) have also studied the impact of silver nanoparticles on

the growth and feeding responses of two lepidopteran pests *S. litura* and *Achaea janata L.* Plant growth and nutrition as well as insect pest tolerance has also been reported to get enhanced through the use of calcium carbonate nanoparticles (Hua et al. 2015). Examples of bio-nano-insecticides include chitosan nanoparticle coated fungal metabolite from *Nomuraea rileyi (F.)* (Chandra et al., 2013) and DNA tagged-nanoparticles (gold, silver, CdS and TiO_2) (Chandrashekharaiah et al. 2015) against *Spodoptera litura*. Others examples include nanoparticles of novaluron against Egyptian cotton leaf worm *Spodoptera littoralis;* but its toxicity was at nearly at par with that of the commercial formulations (Elek et al. 2010). Research in the area of bio-nano-insecticide involves the use of biological (plant) extracts (Sahayaraj et al. 2015) as well as the use of microbes or the extracted bioactive compounds from these species (Kitherian 2017).

17.1.3.4 Nano-fungicides

The versatility of fungal species in causing plant diseases is evident from the fact that these fungi are responsible for causing up to 70% of damage in major crop species (Agrios 2005) and significant crop loss is observed in several crop species like rice, wheat, barley, cotton, groundnut and grapevine (Dhekney et al. 2005). Fungal diseases in plants are generally managed by the use of synthetic fungicides. However, they leave several non-specific effects that destroy beneficial organisms, open the route to undesirable health and safety and several environmental risks (Manczinger et al. 2002). To preserve biodiversity, it has become necessary to explore the strategies and achieve disease management by alternate approaches such as nanotechnology. Several nano-based fungicides, including those that use silver as their active ingredient, have shown promising results.

Select nanoparticles have so far been studied for their role in controlling plant diseases. These include nanocarbon, silver, silica and alumino-silicates. Nanoforms of some commercial pesticides have also been investigated (Kumar et al. 2016). They have assessed the efficacy of nanoforms of Trifloxystrobin 25% + Tebuconazole 50% (75 WG) against the soil borne fungal pathogen *Macrophomina phaseolina* and suggest a positive response towards enhancement of their antifungal activity. Nano-biocide a product prepared by mixing several bio-based chemicals was reported to eliminate fungus *Magnaporthe grisea*, the causal agent of rice blast disease (Gogoi et al. 2009). The design and periodic application of nano-fungicides enable smaller quantities of the fungicides to be used effectively make them resistant to the severe environmental processes that act to eliminate conventionally applied pesticides, i.e., leaching, evaporation and photolytic, chemical hydrolysis and biodegradation. Saharan et al. (2013), evaluated the size and stability of Chitosan and Cu-chitosan nanoparticles with respect to their improved antifungal activity against *A. alternata, M. phaseolina* and *R. solani,* under in vitro conditions. In future the use of nano-fungicides synthesized through a cost-effective technique could lead to the development of bio-hybrid nanocides that will be more environment friendly and effective against multiple fungal pathogens (Abd-Elsalam and Alghuthaymi 2015).

17.1.3.5 Nano-herbicides

Development of improved crop management practices is a continuous process. The intensive use of herbicides for crop improvement in modern agriculture causes environmental problems with toxicity and carcinogenic consequences. The use of current generation of herbicides leads to several environment related problems because of their long term stability, solubility, bioavailability and soil absorption. Further, the movement of these chemical entities to water bodies adversely affects water quality, resulting in negative impact to human species and other biotic life. This has universally initiated efforts to minimize the agro-chemical (herbicide) related environment risks by altering the composition and release dynamics of the herbicides in use (Perez-de-Luque and Hermosin 2013). Use of nano-encapsulated materials, like the use of 2, 4-dichlorophenoxyacetate encapsulated in zinc–aluminium-layered double hydroxide are being developed as alternative herbicide products (Bin Hussein et al. 2005). Additionally, nano-composite materials are being developed, with chitosan and clay as an adsorbent entity, for the herbicides present in aqueous solutions or in a mixture of water and soil (Celis et al. 2012). In another study by dos Santos Silva et al. (2011) alginate/chitosan nanoparticles have been used as a carrier system for paraquat application. It is suggested that this carrier system changes the release dynamics of the herbicide and hence can be further exploited for reducing the negative impacts caused by paraquat. Efforts have also been made to decrease the paraquat herbicide toxicity through the preparation and evaluation of chitosan/tripolyphosphate nanoparticles as carrier systems for this herbicide (Grillo et al. 2014). Some of the products current being developed or used for various agricultural purposes are enlisted in Table 17.1.

17.1.4 Nanoscience and Precision Farming

The advent of GPS (Global Positioning System) and GNSS (global navigation satellite system) have enabled the practice of precision agriculture. Precision farming is a multidimensional technique wherein the scientist use the date obtained from global satellites and remote sensing mechanisms to identify and quantitate varies physical parameters affecting plant growth. The date obtained can also help us to analyze the growth pattern of crops, and their interaction with localized environmental conditions. The date obtained can help precisely indicate the nature of physical vagaries, so that a solution can be applied in time. The data obtained using this technology can then be used to develop maps with spatial variability of various measured parameter (e.g. humidity levels, soil moisture content, terrain features/topography, organic matter content, nitrogen levels, pH, nutrient levels) for optimizing crop productivity (McBratney and Pringle 1999). Precision farming enables maximal output from crops with optimal inputs of irrigation water, type and quantity of fertilizers needed and other agro-chemicals, in a targeted manner. The use of smart sensor systems further enables this technology for enhanced productivity in agriculture by providing accurate information (Cioffi et al. 2004).

Table 17.1 Current nano-products and their uses

S.No.	Product name	Application	Manufacturer
1.	Nano Shield	Nano-based antimicrobial product acts as a fungicide, antibiotic (bactericide), virucide and nematicide. Also useful as a fumigant and disinfectant	Anand Agro Care, Maharashtra
2.	Nano-Virat Yellow	Antiviral, useful for all kind of viral infection in tomatoes and chilly	Futurex Industries, Gujarat
3.	Dabang Yellow	Nano-insecticides. Effective against sucking pests	Futurex Industries, Gujarat
4.	Eco Mites Miticide Nano	Effective against mites. Useful for all crops	Futurex Industries, Gujarat
5.	Tropical nano PHOS	Nanofertilizer	Geetharam Agencies, Kerela
6.	Nano Humic Chelates Crystals	Nanofertilizer	Sanchay Agro Inputs, Maharashtra
7.	Nanofertilizer	Nanofertilizer	Goodwill Rasayan, Rajasthan
8.	Calex (Ca-Mg-S Fertilizer)	Nanofertilizer	Multiline Agro Industries, Maharashtra
9.	Nanomol (S) Micronutrient Fertilizers	Nanofertilizer	Alert Biotech, Maharashtra
10.	Eco PGR	Nanotechnology based plant growth promoter	Futurex Industries, Gujarat
11.	Silver Nanoparticle	For enhancing growth and yield	Intelligent Materials Private Limited, Punjab
12.	Granules and Crystals Gold Nanoparticle	For enhancing growth and yield	Indian Platinum, Maharashtra
13.	NANO-PGR Fruit Special	Growth and yield enhancement	Vision Mark Organic, Maharashtra
14.	Nano zyme Brown	Growth and flowering	Futurex Industries, Gujarat
15.	Nano Humic	Growth	Futurex Industries, Gujarat
16.	Super White Yellow Nano Whitefly Biopesticide	Nano-insecticides against whitefly	Futurex Industries, Gujarat
17.	Thrips Special Nano	Nano-repellent	Futurex Industries, Gujarat
18.	Eco WT Whitefly Special	Nano-insecticide	Futurex Industries, Gujarat
19.	Biolarvicide Nano	Nano-repellents	Futurex Industries, Gujarat
20.	Eco larva Larvicide Nano	Nano Larvicide	Futurex Industries, Gujarat

(continued)

Table 17.1 (continued)

S.No.	Product name	Application	Manufacturer
21.	Emamectin Benzoate 72% nano	Nano-insecticide	ALRChem, Telangana
22.	Nano-Wiper	White fly control nano-pesticides	Annadata Organic, Gujarat
23.	Nano-Tracer	Nano-pesticide. Controls thrips mites	Annadata Organic, Gujarat
24.	Nano Shooter	Nano-pesticide	Annadata Organic, Gujarat
25.	Nano Fungicide – Vanish	Nano-fungicide	Sanchay Agro Inputs, Maharashtra
26.	Nano iron-fortified Mushroon (Patented)	Nano-biofortification	G.B. Pant University of Agriculture & Technology, Pantnagar

At the interface of physical and biological sciences lies the development of novel bio-nano-sensors. The knowledge gained from physical sciences can now be used to develop nanomaterials and nanostructures with unique set of properties that can work in conjunction with biological entities e.g. electrochemically active carbon nanotubes, nano-fibers and carbon fullerenes are used to developed nano-sensors for monitoring processes associated with living systems. In the field of precision agriculture, these nano-sensors can provide accurate information related to various soil parameters, water management status and delivery of various agro-chemicals need for crop development and support. For instance, Normalized difference in vegetative index (NDVI) sensor, also called 'green seeker', is a device which uses nano-based light emitting diodes to generate red and near infrared light. NDVI is commonly used to measure plant health and vigor. It gives an indication of the nitrogen requirement in plants so that the right amount of nitrogen is applied at the right time at right place. For optimizing the use of pesticides an acetyl cholinesterase biosensor has been developed, which works on the assembly of multiwall carbon nanotubes onto liposome bioreactors, for detection of organophosphates pesticides (Yan et al. 2013). The presence of specific viruses and crop pathogens can also be potentially monitored using bio-nano-sensors (Brock et al. 2011). This is amply clear that in the times to come nano-sensor enabled technologies will help the farmer to reduce input costs and make farming a more lucrative option along with reducing the farming carbon foot-print (Rai et al. 2012). Hence, nanotechnology with its diverse potential can enable us to reach the goal of sustainable agriculture.

17.1.5 Advancements in Nano-biotechnology

Nano in agri-biotech is a relatively new field and specific agriculture related compounds are being identified for research as well as up-scaling. Smart delivery systems, leading to safer and more profitable agricultural practices are being targeted,

which can increase productivity without harming the environment, as well as non-target tissues (Hajirostamlo et al. 2015). Time bound or regulated release of agro-chemicals and site-directed delivery of various active molecules have shown promising results in the field of plant disease management, nutrient and use efficiency, targeted DNA delivery etc. Discovery and use of novel elements and compounds for optimizing a host of agricultural practices have been identified, and are being actively researched and up-scaled for industrial production. Intelligent use of mechanical, electronic, thermal, optical and elastic properties of carbon nanotubes (CNTs), have diameter in the range of a few nanometers, have opened up unforeseen possibilities to develop several novel nano-based devices, for the benefit of the farming community. Quantum Dots or QDs are gaining focus due to their biocompatible electronic properties, which may be useful in the development of nano sensors. Bakalova et al. (2004) have reported that the quantum dots possess unique spectral properties, in relation to the plant based organic dyes. These spectral properties entail their use as next-gen flourophores that find potential applications in bio-imaging and development of nano-biosensors. Apart from the fluorescence properties, these QDs also show quantum confinement of charge and possess size tunable band energy (Androvitsaneas et al. 2016). Quantum dot based imaging devices are used for in-vivo imaging of biochemical processes in plants, like nutrient translocation etc. Quantum dots also function as photo-catalysts for energizing the electro-magnetic radiation driven conversion of water into hydrogen as a pathway for solar fuel. Judicious application of these materials like CNTs, metallic nano-delivery systems, quantum dots etc. will definitely usher in an era of next nano-biotechnology based agricultural revolution (Raliya et al. 2013).

As discussed earlier, the optimization and standardization of agricultural processes such as nano-encapsulation of agro chemicals for plant delivery can make the processes environmentally benign and more productive. Although, the nano-biotechnology based improvements in water as well as nutrient uptake efficiency and effects of various nanoparticles on the growth and metabolic functioning of plants show significant variations in terms of dosage, stage of applications etc., still the overall results place a major role of these innovations in optimizing agricultural productivity. Application of nano-composites in plant diagnostics and disease prevention can not only provide better control over fungal and bacterial diseases, but can also provide help in controlling those diseases where so far no specific prophylactic measures are available (Nair et al. 2010).

Although it's very clear that nanotechnology in conjunction with biotechnology, chemical engineering and electronics can provide the basis for the development of a range of new processes and products (Huang et al. 2007); and the current research in finding nano-solutions and alternatives to the current agricultural practices has been going-on for more than a decade now, yet the appearance of nano-based products available to the farmers is not keeping pace with it. The pressing problems requiring nanotechnological interventions include long term sustainability and profitability of agriculture and producing more food from the current varieties by exploiting their full genetic potential. Solving such problems requires multiple inputs. Despite the potential advantages, the number of

commercially available nanotech products in the agricultural sector are still very few. The list of research discoveries and potential innovations seems to be mainly limited to academic sector. Therefore, there is a need to develop greater synergy between the academia and industry, so as to fully exploit the potential of this converging technology (Parisi et al. 2015).

17.1.6 Nano-toxicity and Societal Implications of Nano Products

In the recent past, nano-scale technologies have encompassed almost all spheres of human endeavors and have successfully ushered in a nano-revolution. Different studies on consumer acceptance of nanotechnology based products reflect that the general public opinion is not negative. However, with newer commercial applications of nano-materials being realized, the environmental impact of nano-materials is also expected to increase substantially in future. Despite numerous reports on the potential applications and benefits of nano-materials, there is a substantial gap in the information concerning the environmental and human health implications of exposure to nanomaterials (Fig. 17.3), during manufacturing as well as handling processes and consumer application of various nano-based products.

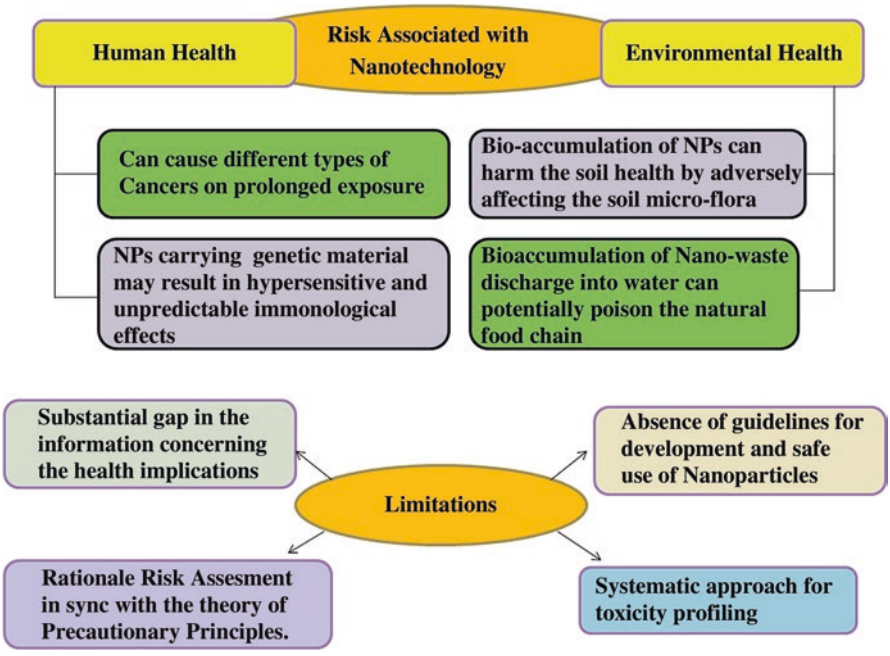

Fig. 17.3 Risks and limitations of nanotechnology in agriculture

An array of nanoparticles have been studied for their ability to induce cellular and immuno-toxicity. Various cell cultures studies, including cancer cell lines, have been used as in vitro toxicity evaluation models, to elucidate the effects of nanoparticles on human health. However, the toxicity data generated by employing in vitro models are conflicting and inconsistent. Nevertheless, a substantial proportion of studies on the assessment of toxicity of nanomaterials to human health and ecosystem suggest that metal nanoparticles exert cytotoxic effect depending on their charge. Nano toxicity may be accelerated due to electrostatic interaction between nanoparticles and biological membranes and their interaction with various cellular metabolites in the cytoplasm (Prasad et al. 2017). Some of the nanomaterials that are acidic in nature lead to a decline in soil pH, thereby affecting the soil microbial diversity.

However, it is difficult to make a generalization regarding the toxicity of nano materials as the toxicity depends on several factors including the nature of the nanomaterial, its size, concentration and the application to which it is being put to. In case of a metal nanoparticle, the toxicity profile is governed by various factors including solubility, binding specificity to a biological ligand, its ability to cross biological membranes etc. Studies suggest that some photo-chemically active nanoparticles like TiO_2, ZnO, SiO_2, and fullerenes, when exposed to light can react with molecular oxygen and generate reactive oxygen species like superoxide radicals through direct electron transfer. This may lead to oxidative stress. On the contrary, there are several reports that suggest that nanomaterials provide protection against oxidative stress (Nayan et al. 2016). In order to clarify this dichotomy, in depth studies related to interactions between cellular ligands and nanoparticles are needed. Moreover, extensive long term research needs to be carried out to understand the ecological impacts (like interaction of nanoparticles with other xenobiotic compounds and their bio-accumulation in the food chain) of the nanoparticles in order to help determine possible adaptive measures.

Reference material related to the risk assessment of various nanomaterials and nanotechnologies, emphasizes the possible direct and indirect social impacts of the use of the compounds (Purohit et al. 2017). However, the volume of information available towards this end is very much limited. Also, the mechanism and methodology to monitor them to support risk governance is lacking (Subramanian et al. 2016). In many ways, nanotechnology is an example of attempted technological-fix to problems that in reality require social, economic and political assessment. It is the need of the hour to inform public at large about its potential advantages and associated risks at each step. Besides, it is also critical to produce a trained future work force in nanotechnology. If we abide by these considerations, a bright and beneficial future can be ushered. Better management of technological innovation in the field of nanotechnology with clearer sustainability objectives, high quality environmental and health risk assessment measures are required, for sustainable use of nanotech products, not only in agriculture but in almost every aspect of human endeavor that is touched by nanotechnology.

17.2 Conclusions

Nanotechnology in agriculture has laid the foundation of novel techniques for improving crop health and translating it to increased yields. Several innovative nano-based agricultural products and technologies are replacing the conventional practices, with the overall objective of attaining higher productivity with limited land resources and being benign for environmental health. However, the full potential of nanotechnology in agriculture is yet to come and the current products represent only the tip of the iceberg. Research is underway to exploit the potential benefits of nanomaterials with respect to the DNA delivery vehicles and production of site selective and reduced dose plant protection products. The future of agriculture lies with the smart delivery systems that will provide integrated and inclusive solutions. For example, devices are being developed for diagnosing plant health issues before these are apparent to the naked eye and can start causing concern to the farmer. Such devises will not only diagnose plant health but will also provide relevant solution through integrated nano-delivery capsules.

Apart from these futuristic applications of nanoscience in the agricultural and food production system, it can also be gainfully used in sectors like food packaging for enhancing the shelf life of the agricultural produce. To tackle the problem of hidden hunger, there is an increasing interest on the application of nanoscience towards selective bio-fortification processes. However, issues related to the cost and scale of production of these novel nanotechnological solutions, as well as their toxicological impacts have to be taken into consideration, before the dream of nano-agricultural can be fully realized. Despite significant information being available on the individual nanomaterials being readied for use in agriculture, the biologically safe level of many of these nanomaterials is still not definable. For instance, while research interest in metal based nanoparticle systems is increasing, the current level of our understanding is not adequate to fully predict their interactions with various bio-molecules inside a living system. Thus the development and application of these wonder nano-compounds has to be dealt in a judicious manner so as to optimize and extract maximum benefit from them, without jeopardizing human and environmental health.

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Root Nodule Development in Model Versus Non-canonical Plants

18

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Abstract

Nitrogen-fixing symbiosis is the most successful metabolism-dependent mutualistic symbiosis on earth that influences global nitrogen cycles. Engineering and improvement of this symbiosis is the major avenue towards sustainable agriculture. Root nodule symbiosis (RNS) is restricted to a monophyletic group of angiosperm order. Collectively this group is called nitrogen-fixing symbiosis (NFS) clade. Tremendous multifariousness exists among the infection mechanism, nodule structure, and nitrogen fixation efficiency among nodules. We have gained significant knowledge about the molecular mechanism of RNS in last two decades, using model legumes *Medicago truncatula* and *Lotus japonicus*. In this chapter, we present the current status of model legumes used to unveil the molecular mechanisms behind the development of nodules and RNS. We further introduce non-canonical legume species that have been used to expand our understanding of the traits associated with RNS in plants. We have also highlighted the extraordinary variations which came up during the evolution of the RNS. This comparative approach will enable us to identify the variable genetic events that rendered NFS to the RNS clade. In turn, this knowledge will hopefully allow us to carefully engineer it in non-fixing crops.

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Symbiosis · Sustainable agriculture · Plant microbe interaction · Nitrogen fixation · Development

18.1 Introduction

Nitrogen fixation symbiosis is the most successful plant metabolism dependent on mutualism, that influences global nitrogen cycle. Given the carbon foot printing of industrial nitrogen fixation through the Haber-Bosch process, and the pollution associated with inorganic nitrogen fertilization, the use of naturally fixed nitrogen in production systems is a *sine qua non* condition for sustainable agriculture. Root nodule symbiosis (RNS) is present among members of the legume family as well as nine additional plant families. Strictly confined to the Rosids I, RNS forming species are restricted to the Fabales (Fabaceae), Fagales (Betulaceae, Casuarinaceae, Myricaceae), Cucurbitales (Coriariaceae, Datiscaceae), and Rosales (Rosaceae, Rhamnaceae, Elaegnaceae, Cannabaceae). Together, they are called the nitrogen-fixing symbiosis (NFS) clade (Fig. 18.1). Legumes and the *Parasponia* sp.

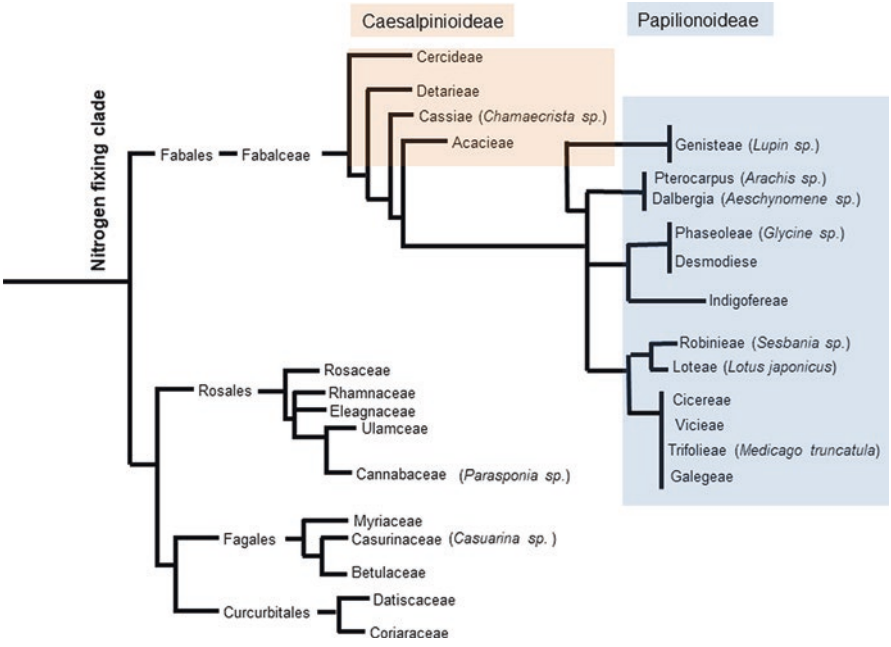

Fig. 18.1 An overview of the phylogenetic relationships among the plants belong to nitrogen fixating clade. The genus names are mentioned for those plants whose detailed descriptions about nodule development are given in the chapter. The subfamilies of the Fabaceae are indicated with coloured boxes. The scientific names of the two model legumes are mentioned

(Cannabaceae) are the only species that can be nodulated solely by gram-negative *Rhizobia*. The remaining of the NFS-clade are nodulated by gram-positive *Frankia*. Only 10 out of the 28 plant families in the NFS-clade can be nodulated either by *Rhizobia* or *Frankia*. NFS-clade evolved ~100 million years ago (MYA) and it is predicted that a single, cryptic evolution event drove the acquisition of the symbiotic N_2-fixation trait (Doyle 2011; Werner et al. 2014). A whole genome duplication ~56 MYA stabilized the nitrogen fixation capacity in the Papilionoideae (Faboidea) subfamily (legume subfamily that contains most of the nitrogen-fixing symbiotic plants). Several pieces of evidence support that multiple gains and losses of RNS gave rise to the present-day scattered distribution of this trait among the NFS-clade (Griesmann et al. 2018; van Velzen et al. 2018). The evolutionary history of RNS explains why so much mechanistic variation can be observed during nodule development and nitrogen fixation, such as the infection process, nodule structure, bacteroid differentiation, and nitrogen fixation efficiency. The most striking differences in the nodule morphologies are summarized in Table 18.1. Taken together, it is evident that tremendous selection pressure worked during the evolution of RNS. On the other hand, multiple losses of this beneficial trait, shows that a negative selection pressure also contributed to shape the NFS-clade.

18.2 *Medicago truncatula* and *Lotus japonicus* as two Temperate Legume Model Systems

In the last two decades, legumes have advanced to the forefront of plant research due to the establishment of two well-supported model, *Medicago truncatula* and *Lotus japonicus* (Oldroyd and Geurts 2001; Riely et al. 2004). Features such as small diploid genome, autogamous genetics, ease of genetic transformation, and short seed-to-seed propagation time made scientists utilize them as models. The genetic and genomics tools available for these species rapidly expanded in the last decade. The gene expression atlases set up for *M. truncatula* (MtGEA: https://mtgea.noble.org/; (Benedito et al. 2008)) as well as *L. japonicus* (http://ljgea.noble.org/) provide a global view of gene expression in all major organ. Recently, the Lotus Base web tool (https://lotus.au.dk/) was released (Mun et al. 2016), which is a comprehensive repository that allows extensive and dynamic exploration of Lotus genomic, transcriptomic data connected with the *LORE1* insertion mutant population (Verdier et al. 2013). Similarly, the *Medicago Tnt1* insertional mutant collection database (https://medicago-mutant.noble.org/mutant/database.php) is connected with the genome browser and MtGEA (Benedito et al. 2008) to facilitate the mutant search. Taken together, information for both models are readily available to allow for gene discovery and functional analyses at a fast pace.

Table 18.1 Diversity of nodulation characteristics among different plant lineages. Fabales (legumes) (Fab), Fagales (Fa), Cucurbitales (Cu), and Rosales (Ro)

					Symbiosome characteristics		Nodule characteristics		
	Clades	Plant species	Microsymbiont species	Invasion type	Symbiosome or fixation thread	Symbiosome shape	Determinant or indeterminant type	Nodule vasculature type	Type of cells in the infection zone
Fab	Inverted repeat lacking clade (IRLC)	*Medicago truncatula*	*Sinorhizobium meliloti*	Infection thread	Symbiosome	Swollen elongated/ Y-shaped.	Indeterminant	Peripheral	Mixture of infected and uninfected cells
	Robinioids	*Lotus japonicus*	*Mesorhizobium loti*	Infection thread	Symbiosome	Non-swollen	Determinant	Peripheral	Mixture of infected and uninfected cells
	Robinioids	*Sesbania rostrata*	*Azorhizobium caulinodans*	Infection thread and crack entry	Symbiosome	Non-swollen	Determinant and indeterminant	Peripheral	Very few uninfected cells
	Dalbergioids	*Aeschynomene indica*	*Bradyrhizobium* sp.	Crack entry	Symbiosome	Swollen spherical	Determinant	Peripheral	Only infected cells
	Indigoferoids	*Indigofera* sp.	*Bradyrhizobium* sp. and others	Infection thread	Symbiosome	Swollen elongated	Determinant, but form secondary clusters of dividing cells at a nodule apex	Peripheral	Mixture of infected and uninfected cells
	Milletioids	*Glycin max*	*Bradyrhizobium japonicum*	Infection thread	Symbiosome	Non-swollen	Determinant	Peripheral	Mixture of infected and uninfected cells

	Genistoids	*Lupinus albus* *Lupinus angustifolius*	*Bradyrhizobium* sp.	Crack entry	Symbiosome	Varies both swollen and non-swollen	A unique subtype of indeterminate nodules called lupinoid nodules	Peripheral	Only infected cells
Fab	Cassiae	*Chamaecrista* sp.	*Mesorhizobium plurifarium, Bradyrhizobium* sp.	Infection thread	Symbiosome and fixation thread	Non-swollen	Indeterminant	Peripheral	Mixture of infected and uninfected cells
Fa	Casuarinaceae	*Casuarina* sp.	*Frankia* sp.	Infection thread	Fixation thread	–	Indeterminant	Central	Mixture of infected and uninfected cells
Cu	Datiscaceae	*Datisca* sp.	*Frankia* sp.	Intercellular(?)	Fixation thread(?)	NA	Indeterminant	Central	NA
Ro	Cannabaceae	*Parasponia* sp.	*Bradyrhizobium* sp. and others	Crack entry	Fixation thread	NA	Indeterminant	Central	NA

All the plants mentioned in this table under Fabales belong to Papilionoideae, which is the major subfamily of Fabales (Naisbitt et al. 1992; Oono et al. 2010; Svistoonoff et al. 2014; Ren 2018)
NA information in not available

18.2.1 Root-Nodule Symbiosis: A Brief Overview

Several years of investigation, mainly concentrated on the temperate legume models *M. truncatula* and *L. japonicus,* have provided a deeper insight into legume-rhizobia symbiosis. The major developmental events of root nodule symbiosis, the bacterial infection at the epidermis and the formation of nodule primordia in the root cortex, are controlled by the host legume. They can be subdivided mainly in (1) epidermal infection; (2) nodule organogenesis; and (3) symbiosome development. As aforementioned and presented in Table 18.1, great variation of mechanisms is observed in every step of symbiosis establishment. In the following section, we briefly mention what we learned from model Papilionoideae legumes and then highlight our current knowledge about the non-canonical pathways for each of the developmental phases.

18.3 Epidermal Infection Events: Using Infection Thread Entry and Nod Factor Signaling

The most common and best-studied mode of infection starts with rhizobia-induced root hair curling – the so called shepherd's hook. The rhizobia get entrapped in the curl and cause the formation of an infection pocket, where bacteria divide and generate micro-colonies known as infection foci (Oldroyd et al. 2011). These are the sites where local hydrolysis of the cell wall takes place and the plasma membrane together with the cell wall invaginates resulting in the intracellular infection threads (IT) that grow towards the base of the root hair, traverse the peripheral cortical cells and eventually reache the inner cortex, where a nodule primordium is forming (Xie et al. 2012). The topology of the IT from the cytoplasmic side is as follows plant plasma membrane, plant cell wall, and glycoprotein matrix containing the rhizobia. The IT propagates as a hollow tube within the root hair cell accompanied by rhizobial cell division inside the glycoprotein matrix. This mode of rhizobial invasion is common among temperate legumes, including *M. truncatula* and *L. japonicus.* Nodulation factors (Nod factor, NF) released by the rhizobia in close proximity to the root hair induce cytoskeletal rearrangements via microtubular reorganization in growing root hairs within 3–10 min upon the exposure (Weerasinghe et al. 2005). Bacterial surface polysaccharides act as additional signaling molecules for the host plant at this stage (Gibson et al. 2008; Oldroyd et al. 2011). Even though the rhizobia lie inside the root hair cells while colonizing the IT, they practically stay outside of the cell as they never encounter the host cytoplasm. Hence, they initially behave like free-living bacteria inside the IT lumen, and then eventually turn into microsymbionts (called bacteroids) after endocytosis once inside the nodule cell, thus becoming surrounded by a plant-derived membrane (peribacteroid membrane). It is interesting to note that in the actinorhizal nitrogen-fixing symbiosis between nonlegume plants from the family Fagales and actinobacteria *Frankia* the nodule development mechanism somewhat resembles the model legumes. For example in *Casuarina glauca* and *Frankia* interaction, the bacteria invade the plant through the IT formation (Svistoonoff et al. 2014).

18.3.1 Molecular Mechanisms in Model Legumes

The first step in the development of symbiosis is the reciprocal exchange of chemical signals between legume roots and the soil borne bacteria *Rhizobium*. Under nitrogen-scarce conditions, the host root secretes specific flavonoids as signals for their cognate micro-symbionts. Flavonoids activate the NOD-cassette in the bacterial genome to induce the biosynthesis and exudation of specific signaling lipochitooligosaccharides (LCOs or NFs)'(Oldroyd 2013). Mutations that render rhizobia defective in the NF production preclude infection of the host legume, and legume mutants that fail to recognize NF are not able to establish the symbiosis. To perceive the NFs, plants utilize the LysM domain (resembling LysM domains of peptidoglycan and chitin-binding proteins) containing receptor-like kinases. Two forms of LysM receptor kinases have been identified. One form has a functional kinase domain and is represented by Nod factor receptor (NFR1) of *Lotus* and LysM receptor kinase 3 (LYK3) of *Medicago*. *Lotus* NFR5 and the *Medicago* Nod Factor Perception (NFP) belong to the second group that lacks a functional kinase domain (Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003; Arrighi et al. 2006). The *Lotus* NFR1 and NFR5 function together as heterodimers or large heterocomplexes and might activate the downstream signaling cascade by trans-phosphorylation of kinase domains. Additionally, an extracellular leucine-rich repeat (LRR) domain-containing receptor kinase SYMRK (symbiosis receptor-like kinase) of *Lotus* as well as the *Medicago* Does not Make Infection 2 (DMI2) are also required for the perception of NF (Endre et al. 2002; Stracke et al. 2002). Biochemically, the concerted action of the kinases (e.g., NFR1-NFR5 in *Lotus*) forms the functional receptor that binds to the species-specific NFs (Broghammer et al. 2012). *Lotus nfr5* and *Medicago nfp* mutants have a more severe phenotype that blocks almost all symbiotic responses, whereas the *Lotus nfr1* and *Medicago lyk3* show a much milder phenotype that retains several of the responses (Radutoiu et al. 2003; Smit et al. 2007). This has been a long-term mystery regarding the epidermal NF response. It has been solved very recently after the discovery of a third part of the NF receptor in *Lotus*: the epidermal LysM receptor (NFRe). *NFRe is* expressed specifically in the root epidermis and controls NF-mediated signaling, via interaction with LjNFR5 (Murakami et al. 2018). Remarkably, *LjNFRe* could not replace *LjNFR1* functionally when expressed under a constitutively (e.g., *pCaMV35S*) or even under its native promoter, suggesting that a complex tri-molecular interplay guides nodule development. To make the symbiosis cost-effective, the nodule numbers must be controlled by the plant. This is accomplished in a number of different ways. Fine-tuning of the NF signaling is very important. The third NF receptor is probably one of the players in nodule number control (Murakami et al. 2018). The second component of the nodule number control mechanism became evident after the discovery of the NF hydrolase gene in *Medicago* (*MtNFH1*). The host plant appears to possess an NF-degradation tool that enables it to control the level of *Rhizobium* infection and nodule development (Cai et al. 2018). Therefore, NF signaling and perception in root epidermal cells is a common theme in most legume-rhizobial interactions. Notwithstanding, the *Lotus nfr1* and *Medicago lyk3* mutants have different

symbiotic phenotypes, which suggests that even though the signaling pathway evolved from the same basic framework, they also took distinct evolutionary trajectories in these two clades of the Hologalegina, which separated circa 50 MYA.

In Medicago, LYK3 interacts with FLOT4 (FLOTILLIN-4, a peripheral membrane protein) and MtSYMREM1 (SYMBIOTIC REMORIN 1, another scaffold protein). Interaction of both MtFLOT4 and MtSYMREM1 with MtLYK3 stabilizes the complex, which prevents endocytosis of the receptor and ensures effective rhizobial entry (Liang et al. 2018). The effective signaling mediated by this receptor complex in the plasma membrane ensures NF-induced peri-nuclear calcium oscillations. These oscillations also require nuclear membrane-localized cation channels, such as CASTOR and POLLUX in *Lotus* while in *Medicago* it is a single inner membrane-localized channel MtDMI1 (Ane et al. 2004; Capoen et al. 2011; Liang et al. 2018). In *Medicago*, a sarco/endoplasmic reticulum Ca^{2+} ATPase (the Ca^{2+}pump, MtMCA8), three cyclic nucleotide-gated channels (CNGC15a, CNGC15b, CNGC15c), and a K^+-permeable channel (DMI1) are involved in the self-sustained Ca^{2+} oscillations. The cyclic nucleotide-gated channels form a complex with the DMI1 in the nuclear envelope, which modulates nuclear Ca^{2+} release (Charpentier et al. 2016). The Ca^{2+}pump and three cyclic nucleotide-gated channels were discovered only in *Medicago*. In *Lotus*, at least three nucleoporins (NENA, NUP85, and NUP133) are located on the nuclear membrane (Oldroyd and Downie 2006). In both legume models, Ca^{2+} oscillations are perceived by a nuclear-localized Ca^{2+}/calmodulin-dependent protein kinase (LjCCaMK and MtDMI3) (Lévy et al. 2004; Mitra et al. 2004). The activation of CCaMK is necessary and sufficient to induce symbiotic processes, as gain-of-function mutations in CCaMK cause spontaneous nodule formation in the absence of rhizobia in both model legumes (Gleason et al. 2006; Tirichine et al. 2006). CCaMK phosphorylates LjCYCLOPS and MtIPD3 (Messinese et al. 2007; Yano et al. 2008), which is essential for the rhizobial entry. Complexation of CCaMK and phosphorylated CYCLOPS induces the expression of NIN (Nodule Inception) (Singh et al. 2014), a transcription factor (TF) that in turn activates another set of specific GRAS transcription factors, Nodulation Signalling Pathway 1 (*NSP1*) and *NSP2* (Kaló et al. 2005; Smit et al. 2005). NIN is probably the most vital TF that controls almost every aspect of nodule development (Marsh et al. 2007; Soyano et al. 2014; Yoro et al. 2014). MtNIN binds directly to the promoter of Cytokinin Response1 (*MtCRE1*) gene and induces cytokinin signaling. Indeed, cytokinin is the best-described downstream factor required for nodule organogenesis (Vernié et al. 2015). In *Lotus,* a dominant active mutant of Histidine Kinase 1 (*LHK1*) generates spontaneous nodule development (Tirichine et al. 2007). Importantly, although there is some mechanistic divergence in the upstream NF recognition module in model legumes, the mechanisms are quite well conserved after the NF recognition event, for example Ca^{2+}oscillations, decoding of the Ca^{2+}signature by CCaMK/DMI3, phosphorylation of cyclops/IPD3 by CCaMK, activation of *NIN* and further cytokinin signalling, even though some evolutionary adjustments have taken place, such as those leading to rather different phenotypes of *cyclops* and *ipd3* mutants. The *Lotus* orthologue *NENA* is not present in *Medicago* (Groth et al. 2010). Remarkably, the *nena* mutant overcomes the lack of epidermal

responsiveness by allowing rhizobia to enter via ethylene-dependent crack invasion (Groth et al. 2010). Further, the rhizobial crack invasion has not been described in *Medicago* mutants up to now (Pislariu et al. 2012). Recently, the complete loss or pseudogenization of *NIN* was shown to be behind the loss of nitrogen fixation by some legumes (Griesmann et al. 2018). However, no difference has been noted in terms of NIN function between the two model legumes. To sum it up, the NF-signalling framework is virtually identical in the two legume models all though some mechanistic divergence is observed during the epidermal bacterial invasion.

18.3.2 An Alternate Mode of Rhizobial Invasion: Crack Entry

Although the invasion through root hair is more common among legumes, an alternative mode of entry is observed for approximately 25% of legume genera, and the frequency of this alternative mechanism is more prevalent in tropical legumes (Sprent 2007). This alternative mechanism of entry is called the 'crack invasion'. In this mode, bacteria infect the root directly between epidermal or cortical cells thus bypassing root hairs. Bacteria entering through crack entry mode may or may not have transcellular IT. Hence, the crack entry mode can be subdivided at least into two different categories, one, entry through cracks followed by dispersion of bacteria via transcellular IT; second, entry through crack and spreading of bacteria via an unknown mechanism. The *dalbergoid* (e.g., *Arachis*) and *genistoid* (e.g., *Lupinus*) legumes and the non-legume *Parasponia* (Cannabaceae) display 'crack-entry', where the rhizobia enter through natural cracks at the lateral root base in an intercellular manner. Indeed, this is a characteristic feature of some subtropical legumes (e.g. *Arachis* sp., *Aeschynomene* sp., and *Stylosanthes* sp.) (Sprent and James 2007). In these legumes, rhizobia directly access the dividing cortical cells for development of their nodule and transcellular infection threads are never formed (Boogerd and van Rossum 1997; Sprent and James 2007; Fabre et al. 2015). In *Sesbania* sp., *Arachis* sp., *Stylosanthes* sp., and *Neptunia* sp., rhizobia employ the epidermal breaches formed by the emergence of lateral roots whereas in the case of *Aeschynomene*, crack invasion occurs at the site of adventitious root emergence. Actinorhizal species of the Rosales order, including *Parasponia,* are infected intercellularly. The bacteria enter the plant root via dissolution of the middle lamella. The infection mechanism in the order Cucurbitales is not known due to the uncultivable nature of the micro-symbiont. The second type of crack invasion has been observed in some legumes, such as *Sesbania rostrata* and *Chamaecrista fasciculata*, in which the bacteria enter through epidermal cracks but eventually the intercellular ITs are formed. A similar situation has been observed in case of *Lotus nena* mutant. Sprent and James (2007) postulated that legume nodules were first initiated from crack infection and subsequently two distinct branches of nodule development evolved from this mechanism, one of which involved transcellular ITs and eventually developing into a sophisticated root hair entry and the other retains 'crack entry' as it is. Further support to this hypothesis came from the nodule formation phenotype of the *Lotus nfr1-1/nfr5-2/snf1* triple mutant, where bacterial

invasion occurs through cracks. This suggested the crack infection is indeed the ancestral stage of bacterial invasion (Madsen et al. 2010).

18.3.3 Nod Factor-Independent Nodule Development

NF-mediated chemical crosstalk is a key determinant for RNS in model legumes. However, there is a group of photosynthetic *Bradyrhizobium* strains (*ORS278 and BTAi1*) that are able to induce the formation of nitrogen-fixing nodules despite the absence of NF biosynthesis (the canonical *nodABC* cluster) genes in the bacterial genome (Giraud et al. 2007). Both *ORS278* and *BTAi1* generate nodules on tropical aquatic legumes that belong to the *Aeschynomene* genus, namely *A. sensitiva* and *A. indica*. In these *Aeschynomene* legumes, bacteria enter through 'crack invasion'. Interestingly, a *nodB* deletion in the *Bradyrhizobium* strain *ORS285* (wild type harbors *nodABC* genes) causes it to lose its ability to infect broad host range, still it is able to induce nodules in some *Aeschynomene* species (Giraud et al. 2007). These observations indicate that certain *Aeschynomene* species are capable of initiating the symbiotic association via two routes: the conventional NF-mediated pathway and alternate signaling pathway independent of NF. In genus Aeschynomene, *A. indica* shows NF-independent nodulation while *A. afraspera* is characterized by NF-dependent nodulation. *ORS285* does not produce NF during nodule development in *A. indica*. Moreover, artificial induction of NF during this symbiosis delays nodulation, which suggests that NFs have an adverse effect on NF-independent nodule development. *CCaMK*, *SYMRK* and *HK1* orthologues are reported to be required for efficient NF-independent nodulation in *A. evenia-Bradyrhizobium* symbiosis (Fabre et al. 2015). This indicates that the core signaling components are conserved during the establishment of both NF-dependent and NF-independent symbiotic programs.

18.3.4 The Role of Rhizobial Secretion Systems During Nodule Development

Like all bacteria the symbionts contain all the molecules that can induce a defense response in plants. The symbiotic relationship may shift from being mutually beneficial to pathogenic if the plants lose the ability to regulate the total number of nodules. The same may happen if the rhizobia form nodules that do not fix nitrogen at all or fix nitrogen in amounts below a certain threshold (Herridge and Rose 2000; Kiers et al. 2003; Pahua et al. 2018). The whole genome sequence analysis of several symbiotic rhizobial strains revealed the presence of secretion systems like those of pathogenic bacteria which they use to deliver effector proteins in the host cytoplasm, and combat the host's defense. These mechanisms include type III (*T3SS*), IV (*T4SS*), and VI (*T6SS*) secretion systems. Transfer of effector proteins to host cells functions as a key determinant of host specificity. Notwithstanding, knowledge about genetic basis of these processes and molecular function of associated proteins

is still in its infancy (Masson-Boivin et al. 2009). Several studies on rhizobia mutants that lost the secretion system(s) showed both positive and negative effects on the symbiosis (Deakin and Broughton 2009; Clúa et al. 2018). Comparative genome analysis of 48 *Sinorhizobium* strains revealed that they can contain either the *T3SS* or *T4SS* or *T6SS*, but typically only one of them gets involved in the symbiosis in a given strain (Sugawara et al. 2013). These effector proteins could potentially have a function by promoting nodule formation, disrupting autoregulation of nodule number (AON), or suppressing the plant's immune response during the invasion (Sugawara et al. 2013). In the following section, we summarise the role of different secretion systems during nodule development.

18.3.4.1 The Type III Secretion System (*T3SS*) or the Injectosome

The T3SS is made up of about 30 different proteins and carries out secretion of type III effector proteins (T3Es) (Ghosh 2004; Tampakaki 2014). Approximately 50% of proteins constituting this secretion system channel are conserved in most T3SSs. These proteins are generally clustered in a 22–50 kb pathogenicity island in the bacterial genomes (Tampakaki 2014). The T3SS complex spans the bacterial inner and outer membrane as well as the hosts' membranes and mediates protein delivery from the bacterial cytoplasm directly into the host. On the other hand, the majority of effector protein-encoding genes are scattered throughout the bacterial genome but a few may be found in the regions flanking the pathogenicity island (Lindeberg et al. 2008). The genes specifying the rhizobial *T3SS*s are called *rhc* (for *Rhizobium* conserved) (Gazi et al. 2012). The first report that the T3SSs can induce nodules came from the study of symbiosis between *Bradyrhizobium elkanii* and soybean (Okazaki et al. 2013). *B. elkanii* can trigger nodule morphogenesis in soybean natural *nfr* mutant *En1282*. Interestingly, deletion of the *T3SS* impaired nodule development in *En1282*. Soybean flavonoids induce *B. elkanii* T3SS, which then injects several effector proteins into the host cell. These effector proteins activate the NF signaling pathway by passing the requirement for the NF-receptor. Hence, T3Es work as an alternative of NF to induce nodulation signalling pathway (Okazaki et al. 2013). Further, a systematic investigation has been conducted on the *Aeschynomene* species that shows the NF-independent nodulation. *B. elkanii* can generate nodules on *A. indica* root all though the nodules are ineffective. Detailed characterization revealed that all though the rhizobia in this example cause nodule formation they proliferate only in the intercellular space of the nodule. Further, a screening has been undertaken using 72 different rhizobial strains that belong to *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Beta-rhizobium* to check their ability to induce nodules on *A. indica* roots. Interestingly, only strains that belong to *Bradyrhizobium* genus were able to elicit nodules. All *Bradyrhizobium* strains that can generate nodules possess the T3SS but not all the *T3SS*-containing *Bradyrhizobium strains* can generate nodules. This suggests that the *T3SS* and its conserved effector modules are necessary for nodule formation. The same group also evaluated *t3ss* deletion mutants of the *Bradyrhizobium* strain *ORS285*. Surprisingly, the *t3ss* deletion did not affect nodulation of *A. indica* by *ORS285*. This result indicates two alternative modes of infection: the first one is the NF-independent and type III-dependent in which the

nitrogen fixation efficiency is low, and the other mode is NF-independent and type III-independent where the nitrogen fixation efficiency is high. Further interaction of NF-dependent nodulation and the T3SS has been observed in several cases. It is either synergistic or antagonistic depending on the host plant. The classical example is *Sinorhizobium fredii* strain *NGR234*-mediated nodule development in diversely distinct legumes. Abolition of the *T3SS* in this strain leads to different phenotypes depending on the host. It showed no significant effect on cowpea (*Vigna unguiculata*, phaseolid), whereas it affected negatively *Lablab purpureus* (phaseolid), and two millettioid species, *Flemingia congesta* and *Tephrosia vogelii*. At the same time the *t3ss*-deprived strain exhibited a positive effect with *Pachyrhizus tuberosus* (phaseolid), *Crotalaria juncea* (genistoid), and *Leucaena leucocephala* (Mimosoideae) (Skorpil et al. 2005; Kambara et al. 2009; Nelson and Sadowsky 2015). Taken together, it is now well established that the rhizobial T3SS can hijack the nodulation signaling pathway. It is also clear that a complex interplay takes place in the natural rhizosphere between the NF-mediated and the T3SS-mediated pathways.

Until now, only a few T3Es have been reported as crucial for nodule development. Interestingly, some of these effectors are found to be key players in modulation of plant immune signalling (Miwa and Okazaki 2017). A novel E3 ubiquitin ligase (NEL) domain effector with LRR, the NopM (Nodulation outer protein M) of *Rhizobium sp. NGR234* is required for efficient nodulation of *Lablab purpureus*. A point mutation in *NopM* that impairs its enzymatic activity causes a reduced nodule number compared to the wild-type strain (Xin et al. 2012). Some plant genes that restrict nodulation have been associated with the recognition of rhizobial effector proteins. The soybean alleles *Rj2* and *Rfg1* encode an R-protein of the Toll-Interleukin Receptor-Nucleotide-Binding Site-Leucine-Rich Repeat (TIR-NBS-LRR) that restricts nodulation by *Bradyrhizobium japonicum* (*USDA122*) and *Sinorhizobium fredii* (*USDA257*), respectively (Yang et al. 2010). This indicates that an unidentified rhizobial effector(s) is recognized by this R protein, which triggers Effector-Triggered-Immunity (ETI) and prevents nodule development. Another soybean protein, called thaumatin-like *Rj4*, restricts nodulation by many strains of *B. elkanii*. *Rj4* is required for host defense and a wide range of developmental processes and its loss of function induced by the CRISPR/Cas9 system enabled nodulation by the incompatible rhizobia *B. elkanii* (USDA61) (Tang et al. 2016)

18.3.4.2 Other Secretion Systems

The T4SS is divided into three sub-families (Cascales and Christie 2003): (a) conjugation systems that translocate DNA substrates intercellularly from bacteria to host; (b) effector translocator systems that function like the T3SS; and (c) DNA release/uptake systems which translocate DNA to or from the extracellular milieu. The T4SS-b is functionally analogous to the T3SS in mediating protein translocation but has a distinct evolutionary origin. They share similar core proteins to form the main channel. In rhizobia, the T4SS-b exhibits strong homology to the *Agrobacterium* VirB/VirD4 subunits. The T4SS-b core structure consists of 12 proteins, VirB1-B11 and VirD4. *Agrobacterium tumefaciens* uses T4SS-b for translocation of both,

T-DNA and effector proteins (Kuldau et al. 1990; Zupan and Zambryski 1995). So far, only three different rhizobium have been reported to possess the functional *T4SS-b* (Nelson and Sadowsky 2015). Similar to the T3SS, the T4SS-b can also exert either a positive or negative impact on symbiosis. Mutation in the *Mesorhizobium loti* R7A-T4SS or in any of the effector that it transports leads to reduced number of nodules on *Lotus corniculatus*. However, the same deletion allowed *M. loti R7A* to form functional nodules on *L. leucocephala* (Hubber et al. 2004). A defective *T4SS-b* in *Sinorhizobium meliloti KH46c* resulted in an approximately 50% reduction in nodule number on *M. truncatula* A17 but it did not have a significant effect in *M. truncatula F83005-5* (Sugawara et al. 2013). Interestingly, with induction of NFs, plant-secreted flavonoids activated *tfeA* and *virG* transcription in rhizobia (Nelson et al. 2017). *S. meliloti* and *S. madicae* both secrete the effector protein TFeA.

The third secretion system that has been implicated in root nodule development is the T6SS. Deletion of the T6SS secretion system (the *imp* gene cluster) in *R. leguminosarum* enables this strain to form functional nitrogen-fixing nodules on pea (Filloux et al. 2008). This establishes a negative role of the T6SS in the root nodule symbiosis. Whether the T6SS imparts any positive effect on root nodule development is yet to be investigated.

18.4 Nodule Organogenesis

Diverse types of nodules are formed by legumes in association with a wide range of rhizobia. According to their growth pattern, nodules are typically classified as either determinate or indeterminate (Hirsch 1992; Sprent 2007), which are exemplified in the model legumes. Indeterminate nodules have a persistent apical meristem whereas determinate nodules have a non-persistent peripheral meristem only present during organ development. In *M. truncatula*, development of the indeterminate nodules starts from the inner cortex and pericycle of the root (Xiao et al. 2014). In contrast, determinate nodules of *Glycine max* are formed from the outer cortical layers (Turgeon and Bauer 1982) whereas those in the other model legume *Lotus* emerge from the middle cortical layers (Ndoye et al. 1994; Madsen et al. 2010). In turn, the determinate nodule of peanut (*Arachis hypogaea*) originates from the root pericycle with the endodermal, cortical and epidermal layers shredded in the mature nodule (Tajima et al. 2008). Hence, it is probably not appropriate to classify nodules simply by the presence of a meristem in the mature nodule. They should rather be further subdivided. Table 18.1 features the differences in nodule development among different legumes. The readers should note that this is just a simplified way to describe the whole diversity of nodule types characterised so far. Figure 18.2 summarizes the probable path of evolution of different types of nodule development.

18.4.1 Different Types of Root Nodules

Indeterminant nodules retain the meristem in the nodule apex, hence considered as modified roots, whereas the vascular strand anastomosis and absence of meristem in

the nodule apex place determinant nodules one step ahead in evolution. In the following section we are going to describe different nodule types and our knowledge about signalling pathways that govern determinant and indeterminant nodule development.

18.4.1.1 The Indeterminate Nodules: A Lesson from *Medicago*

The typical *Medicago* root contains the following - (i) outermost single epidermal layer, (ii) four-to-six cortical cell layers (iii), single layer of endodermis; (iv) pericycle. Nodule development involves de-differentiation and re-differentiation of the root cortical cells (Fig. 18.2). In *Medicago*, the development of both organs, the lateral root and the nodule, starts with cell divisions in the pericycle, endodermis, and cortex (Herrbach et al. 2014; Xiao et al. 2014). However, the development of nodule involves more action in the middle and inner cortical cells. Within 70 h post-inoculation (hpi), a typical nodule primordium is already formed. The primordium is composed of a developing multi-layered meristem derived from the middle cortical layer, an infection zone with eight cell layers derived from inner cortical cells, and nodule periphery with six to eight cell layers derived from the endodermis and pericycle. At 80 hpi, vascular bundles are established at the periphery and bacterial release occurs in the dividing inner cortical cells. The use of *AtCyclB1.1::GUS* (cell division marker) and *MtCCS52A::GUS* (endo-reduplication marker)-expressing *Medicago* lines show that the inner cortical cell division almost ceased around 70 hpi while the endoreduplication of the cortical cells started around that time. The

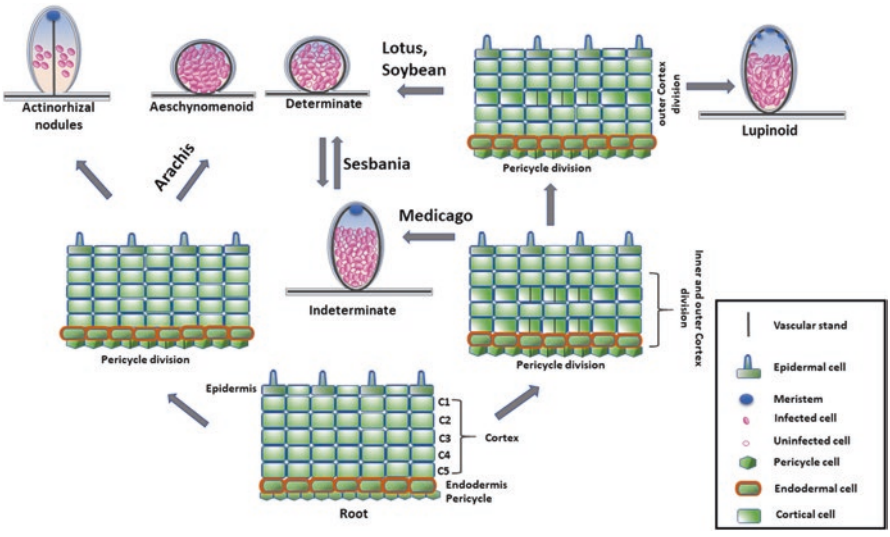

Fig. 18.2 Schematic illustration of diversity in nodule development: It shows the site of cell division during nodule primordia formation across the nitrogen fixating clade. The typical root structure was drawn according to Xiao et al. (2014). Lines inside cells represent their dividing state. The orange borders around endodermal cells are Casparian stripes. The genus names indicated are those for which detailed description of nodule development is given in the chapter

endodermis-derived cells maintain expression of endodermal gene (*AtCASP1::GUS*) in the nodule primordium, which suggests the complete de-differentiation takes place only in the root cortical cells, whereas endodermal cells switch directly from one differentiated cell type into another, a process known as trans-differentiation (Xiao et al. 2014). In the mature *Medicago* indeterminate nodule, due to the presence of the persistent apical meristem, infected cells, continuously added from the nodule meristem give rise to the typical cylindrical shape. In a typical 35-day post inoculated (dpi) fully mature nodule, four discrete developmental stages can be seen: (i) the apical meristem; (ii) the invasion zone, where bacterial endocytosis and bacteroid differentiation take place; (iii) the nitrogen fixation zone, where nitrogen fixation and metabolite exchange take place – this region contains infected cells with leghemoglobin, which gives the functional nodule its remarkable pink color; and (iv) the senescence zone, only present in older nodules, absent in 21 dpi nodules. The indeterminate nodule can be branched or not. Recently it has been shown in *Medicago* that excessive NF amount can induce the nodule to branch (i.e., form coralloid structures) and induce higher nitrogen fixation rates, whereas the optimum NF amount induces the formation of a cylindrical nodule (Cai et al. 2018).

18.4.1.2 The Determinate Nodule Organogenesis

The fundamental characteristic of the determinate nodule is its ephemeral meristem and absence of the developmentally-defined tissue gradient in the mature nitrogen-fixing nodule. Nodule determination is thought to have evolved from the primitive indeterminate nodule (Sprent and James 2007). The biggest support for this hypothesis is the presence of both nodule types on *Sesbania rostrata* roots. Which nodule type develops depends on root growth conditions. However, *Sesbania* determinate nodules are somewhat of the hybrid type – like the indeterminant nodule, they start developing with a transient coexistence of developmental zones, and then nodule meristem activity stops, and the mature nodule becomes determinate. Furthermore, Ag^+ ions, which have been widely used as inhibitors of ethylene perception, can convert determinate nodules into indeterminate once (Fernández-López et al. 1998). Recently, the concept of nodule determination has been revisited (Ren 2018); nodule development in *Indigofera* (an outgroup of the millettioids) and *Tephrosia* (millettioid) has been reclassified from indeterminate to determinate. Hence, apart from the classical determinate nodule formation of soybean and *Lotus*, several categories of non-canonical determinate nodules are present in nature. Determinate nodules probably originated twice in natural history, once in the Robinioids and Milletioids clade where they form desmodioid type determinant nodules and once in the Dalbergioids clade, which form aeschynomenoid nodules (Sprent 2007). The major difference between desmodioid and aeschynomenoid nodules is that the former contains a mixture of infected and uninfected cells whereas in the latter only infected cells are present. Unusual desmodioid nodules infrequently contain a cluster of dividing cells in the mature nodule due to mitotic reactivation of the nodule cells. This differs from the indeterminate nodule meristem, since the canonical indeterminate nodule meristem differentiates from the root middle cortical cells and maintained throughout its life cycle (Xiao et al. 2014). Hence, the spherical or cylindrical

nodule morphology is not a hallmark to discriminate between determinate and indeterminate nodule types. Rather the cell ontology of the meristem determines the nodule types. Milletioid nodules of *Indigofera* and *Tephrosia* are good examples to illustrate this complexity.

18.4.1.3 Lupinoid Nodules

Lupinus (genistoid) forms lupinoid nodules. The development of the lupinoid nodule displays some features that are peculiar to indeterminate nodules, while others are typical for the determinate type. Lupinoid nodule development starts from the outer cortical cells, similar to determinant nodules (Łotocka et al. 2000). The early phase of lupinoid nodule development resembles to that of *Glycine*. The infected central tissue does not show typical zonation found in indeterminate nodules, and does not have uninfected interstitial cells – instead, the basal-laterally located meristematic cells contain the bacteroids (Golinowski et al. 1987). Early infection varies in different lupine species, but the IT is almost never formed irrespective of the variation in nodule morphology (Tang et al. 1993; Łotocka et al. 2000; González-Sama et al. 2004). An indeterminate-like feature of lupin-nodules is that the infected zone originates from the division of a single infected outer cortical cell. This process is common to all *Lupinus* species studied (Łotocka et al. 2000). After infection, bacteroids divide inside the infected cells and the infected cells remain mitotically active. Mitotic activity of the infected cells gives rise to the distribution of the symbiosomes equally between the daughter cells (Gonzalez-Sama et al. 2004). This is a typical feature of lupinoid nodules. Initially the nodule meristem surrounds the central infected cells circularly and later the meristem is concentrated as fragmented parts in the nodule apex (Łotocka et al. 2000). Recently, an extensive transcriptomic analysis was carried with roots and nodules of three closely-related lupin species (*L. albus*, *L. luteus*, and *L. mariae-josephae*) inoculated with compatible, incompatible, or partially compatible *Bradyrhizobium* strains (Keller et al. 2018). Differentially expressed gene analysis provided new insights into the lupin response to symbiotic compatibility. Symbiont compatibility greatly influences overall nodule transcriptome, such as expression of genes related to hormone, defense, primary and specialized metabolism and general physiology etc.

18.4.1.4 Aeschynomenoid Nodules

Aeschynomenoid nodules are formed by several *Aeschynomene* legumes, including *A. indica*, *A. afraspera*, as well as other pterocarpus species, e.g. *A. hypogaea*. Aeschynomenoid nodules contain a large central infected zone surrounded by a thin uninfected nodule cortex which subsumes vascular bundles (Lavin et al. 2001). In case of *A. indica* and *A. afraspera* nodules, (Łotocka et al. 2000) vascular bundles are connected to the vasculature of the primary root, whereas the nodules are systematically attached to the lateral root (Bonaldi et al. 2011). This two species differ from each other in terms of their dependency on NFs for symbiosis initiation with photosynthetic bradyrhizobia, bacteroid differentiation, and nodule organogenesis. The mature nodules of *A. indica* contain a single, perfectly spherical bacteroid per symbiosome in contrast to younger symbiosomes harbouring multiple bacteria in

A. afraspera. *A. afraspera* nodules are unique by the presence of two distinct infection zones: (A) a central infected tissue filled with infected cells containing differentiated bacteria, singly enclosed in symbiosomes; (B) an outer tissue at the top of nodules made up of a few giant infected cells. The latter contains a network of tubular structures with undifferentiated bacteria enclosed. The bacteria remain rod-shaped and elongates as the nodule ages (Bonaldi et al. 2011). In case of *A. hypogaea*, the nodule development starts from the pericycle opposite to the protoxylem poles. In this species, cortical and epidermal tissues of the root shed from the mature nodule and an endodermal layer is absent in the mature nodule (Boogerd and van Rossum 1997). In addition, the nodule surface is protected by a thin peridermal layer containing lignin and suberin (Tajima et al. 2008).

18.4.1.5 Actinorhizal Nodules

Legumes and *Parasponia* (syn. *Trema*, Cannabaceae) form nodules upon interaction with rhizobia while other nitrogen fixing plants in the NFS clade develop nodules with *Frankia* (Fig. 18.1). Interestingly, legume nodules mainly initiate by cell division in root cortex (sometimes pericycle). The vascular bundles are located in the periphery of the nodule. Nodule development in actinorhizal plants including *Parasponia* begins with the division of the root pericycle. Therefore, the actinorhizal nodules can be considered as modified lateral roots without the cap. Nodules of this type usually have coralloid structures with a central vascular bundle. In such nodules, layers of superficial periderm cells cover the central infected cortical cells. This is sometimes disrupted by lenticels. The different types of the Actinorhizal nodules have been summarized in Table 18.1.

18.4.2 Molecular Mechanism Behind Nodule Organogenesis

Nothing is known about how indeterminate nodules maintain their persistent meristem, and how the determinate nodules lose or reactivate it. It is well-accepted that root developmental programs are co-opted for nodulation (Franssen et al. 2015). Several markers are expressed in the quiescent center (QC) of the root apical meristem of *Arabidopsis*, such as WUSCHEL-RELATED HOMEOBOX (*WOX5)*, QC marker 25 (*AtQC25*) and SCARECROW (*SCR*). Homologs of these genes are also expressed in *Medicago* nodule apical meristem (Sabatini et al. 1999; Łotocka et al. 2012). Further, expression analysis shows that several *Medicago* orthologues of *Arabidopsis* PLETHORA (*PLT1–4)* genes are expressed in the nodule meristem. Nodule-specific downregulation of *MtPLT3* and *4* impede the nodule development (Franssen et al. 2015). Mature *Medicago* nodule meristem contains two types of cell; the central nodule meristem and multiple layers of the nodule vascular meristems (Osipova et al. 2011, 2012; Couzigou et al. 2013; Roux et al. 2014; Franssen et al. 2015). The nodule vascular meristems (NVM) are derived from the root pericycle and endodermal cell layers (Couzigou et al. 2012; Xiao et al. 2014). The Nodule central meristem (NCM) is derived from the root cortex. It has been shown that NVM is characterized by a high auxin response and NCM is

characterized by a higher cytokinin and a lower auxin response. Some of the QC markers mentioned earlier are expressed differentially in the NCM versus NVM in *Medicago* (Franssen et al. 2015). *NOOT* and *COCH* genes are recruited to repress root identity in the symbiotic organ. In indeterminate *Medicago* nodules, loss of NODULE ROOT1 (*MtNOOT1*) and its paralog NODULE ROOT2 (*MtNOOT2*) results in complete loss of its nodular identity and generates roots from the nodule apex (Couzigou et al. 2012; Magne et al. 2018b). In case of determinate nodules, the vascular strands are connected at the nodule apex and do not retain meristem. This indicates that identity of nodular tissues is better controlled in determinate types over their indeterminate counterparts. Interestingly, loss of the orthologue of *MtNOOT1* gene from Lotus (*LjNBCL1*) leads to generation of an ectopic root. This suggests that the same pathway is recruited to maintain both type of nodule identity (Magne et al. 2018a). This also demonstrates that NBCL function in the maintance of nodule vacular bundle identity are conserved in both indeterminate and determinate nodules.

Furthermore, how the root cortex becomes activated downstream to NF signaling is not clear. At first, NF is perceived in the root epidermis (Vernié et al. 2015). How does the NF signal activates the middle or inner cortical cell layer (depending on the nodule type), keeping several outer layers of the root cell unaffected? Several parallel studies highlighted auxin or cytokinin as direct players downstream to the NF signalling. The primary cell divisions coincide with the position of auxin signaling in cortical cells. Cell division initiation also correlates with the presence of an auxin spike, which has been determined through auxin reporter lines in species forming indeterminate (Mathesius et al. 1998; van Noorden et al. 2007; Breakspear et al. 2014; Ng et al. 2015) as well as determinate nodules (Takanashi et al. 2011). By the same token, root flavonoids are necessary for nodule initiation in *M. truncatula* and they act via regulating auxin transport (Wasson et al. 2006).

Specific overexpression of *NIN* either in the epidermis or in the cortex promotes spontaneous nodules in *Medicago*. Additionally, NIN binds directly to the promoter of *CRE1* (*cytokinin receptor*) and induces cytokinin signalling. Again, overexpression of *NIN* or a dominant active mutation in *LHK1* (*Lotus* cytokinin receptor) is sufficient to induce formation of spontaneous nodule-like structures (Soyano et al. 2013). Taken together, these observations suggest that cytokinin is the best described downstream factor involved in spontaneous nodule organogenesis (Vernié et al. 2015). Thus, how auxin and cytokinin ratio balances nodule organogenesis and infection still needs to be determined. Recently, a high cytokinin to auxin ratio has been determined in soybean nodules ratiometrically (Fisher et al. 2018). Overall, the notion is that more cytokinin and less auxin is a condition necessary to initiate the nodule development.

NF signalling in the epidermis initiates nodule organogenesis in the cortex. Concerted action of the epidermal bacterial infection and cell division in the cortex promotes functional nodule development. Independent lines of evidences suggest that epidermal bacterial infection and cortical nodule organogenesis are uncoupled. Spontaneous nodule development by several plant mutants indicate that the nodule

developmental program can be installed without the presence of symbiotic bacteria. Over-expression of any of the following symbiotic receptor-like kinases, SYMRK, NFR5, or NFR1, from a strong ubiquitin promoter led to spontaneous nodules on *L. japonicus* transgenic roots. The effect was more pronounced in SYMRK overexpression in both *Medicago* and *Lotus* (Wasson et al. 2006; Ried et al. 2014; Saha et al. 2014). Release of an auto-inhibitory domain or a phosphomimetic version of Ca^{2+}/Calmodulin-dependent protein kinase (CCaMK) (DMI3 in *M. truncatula*) results in auto-activation of the nodulation signalling pathway and hence generation of spontaneous nodules (Gleason et al. 2006; Tirichine et al. 2006). Nuclear calcium spiking induces CCaMK-mediated phosphorylation of Cyclops/IPD3, which then acts as a transcriptional activator. A de-regulated version of Cyclops leads to spontaneous nodule formation independent of CCaMK (Singh et al. 2014). Direct involvement of cytokinin in the root nodule organogenesis was demonstrated by a gain-of-function mutation in the cytokinin receptor gene *LHK1* in *Lot*us that led to the initiation of nodule formation in absence of rhizobia (Tirichine et al. 2007). The opposite phenotype was observed in loss-of-function mutations of *LHK1* and its *M. truncatula* orthologue *CRE1* (Murray et al. 2007; Plet et al. 2011). NIN and *CRE1/LHK1* act in a feed-forward loop and are required for the gain-of-function phenotype of *LHK1*. Constitutive expression of NIN triggers ectopic cortical cell division leading to the formation of small bumps similar to a nodule primordium (Schauser et al. 1999; Tirichine et al. 2007; Suzaki et al. 2012; Soyano et al. 2013; Vernié et al. 2015). Further, spontaneous nodule development has been observed by the expression of deregulated forms of CCaMK in case of *Parasponia* and Aeschynomene legume *A. evenia* (Den Camp et al. 2011; Fabre et al. 2015). Taken together, it is suggested that epidermal bacterial infection (whether crack or IT) and cortical nodule organogenesis (determinant, indeterminant, or aeschynomenoid) are uncoupled.

18.5 Symbiosome Development and Nitrogen Fixation

'Symbiosome' is an organelle like structure present in the cytoplasm of infected cells. Endocytosis of the bacteria and generation of symbiosome are significant step forwards by the symbiotic plants. This is absent in primitive Caesalpinioideae legumes *Chamaecrista* sp. and non-legume *Parasponia* sp. Rhizobia are retained inside a modified infection thread called 'fixation thread' without forming the organelle like structure 'symbiosome' in primitive legumes, whereas modern Papilionoideae legumes contain symbiosome. Symbiosome is the ultimate evolutionary innovation which acts as a selectively permeable physical barrier to control an efficient exchange of carbon and nitrogen. Hence generation of symbiosome increases the efficiency of the symbiotic nitrogen fixation (Sprent 2007 and Limpens et al. 2005). In determinate nodules, e.g. *L. japonicus*, symbiosomes usually contain two or more bacteroids of similar size, whereas in indeterminate nodules, e.g. *M. truncatula,* symbiosomes contain a single swollen bacteroid that can be shaped pleomorphic.

18.5.1 Symbiosome: The Ammonium Exporter

In *Medicago*, rhizobia start infecting the dividing root cortical cells 80 hpi (Xiao et al. 2014). Specific dissolution of the cell wall by plant pectate lyase is essential for the endocytosis of the rhizobia (Xie et al. 2012). How the endocytosis of rhizobia takes place in molecular terms is not known. Some membrane microdomain-forming proteins such as Flotilin-like peptide (soybean and pea nodules), MtSYMREM1, and GmFWL1 have been detected in the symbiosome membrane (Panter et al. 2000; Saalbach et al. 2002; Lefebvre et al. 2010; Clarke et al. 2015). Some of these proteins are required for it invagination, which indicates they may be involved in bacteroid endocytosis. The only molecule known to participate in during the endocytosis of rhizobia is DMI2. Knocking out *DMI2* generates enlarged ITs without endocytosis (Limpens et al. 2005; Den Camp et al. 2011). After endocytosis, the bacteria become enclosed in the plant cytoplasm by an inside-out plant plasma membrane-derived modified membrane. The bacteria after endocytosis and differentiation to endosymbiotic status are called bacteroids. The plant-derived membrane enclosing the bacteroids is called peribacteroid or symbiosome membrane (PBM or SM). The unit of bacteroids and PBM functioning together during nitrogen fixation is called the symbiosome (Goodchild and Bergersen 1966; Robertson and Lyttleton 1984; Roth and Stacey 1989). Endosymbiotic bacteroids completely shut down many cellular and metabolic activities including downregulation of genes related to ammonium metabolism and transport in both nodule types (Barnett et al. 2004; Capela et al. 2006; Chang et al. 2007; Karunakaran et al. 2009). Remarkably, bacteroids become auxotrophic and dependent on the plant for the branched chain amino acids (leucine, isoleucine, and valine). Therefore, supply of these amino acids by the plant is essential for the bacteroid development and nitrogen fixation by the bacteroids (Prell et al. 2009). Induction of *nif* and *fix* gene expression in bacteroids enables them to reduce N_2 to ammonia. *The fix* regulon controls the expression of *nif* genes, which coordinate nitrogen fixation. FixL is an oxygen sensor in *Sinorhizobium meliloti* -under low oxygen concentration, which is created in the cytosol of infected nodule cells by the action of the plant oxygen binding protein leghemoglobin. FixL phosphorylates FixJ, leading to FixJ activation under this hypoxic condition. Activated FixJ phosphorylates nifA, which in turn activates the nitrogenase subunits *nifHDK*. Apart from the nitrogenase subunits, the *Fix* operon also codes for the high-affinity terminal oxidase (*fixNOQP),* which is critical for microaerobic respiration of rhizobia inside the nodule (Barnett et al. 2004). The molecular players may differ but the central theme of activation of nitrogen fixation inside the bacteroids is fairly conserved in all nodule types. Bacteroids also require an energy source to carry out nitrogen fixation. The general consensus is that dicarboxylates, e.g. malate, succinate, and fumarate are the major carbon source supppied to the bacteria by the plant (Mitsch et al. 2017). In return, bacteria provide fixed ammonia to the plant. Ammonia is generated by the di-nitrogenase enzyme and then probably diffuses out to the symbiosome space (the space between bacteroids and PBM).The symbiosome space is an acidic compartment with pH values around 4.2–5.5). Due to the acidic environment the ammonia gets protonated and converted

into ammonium ion in order to be transported to the plant cytosol and assimilated into amino acids via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway (Udvardi and Poole 2013).

18.5.2 Signalling Pathway Leads to Symbiosome Formation

The nature of signalling pathway leading to symbiosome formation is almost a black box. Several unique adjustments are required during symbiosome development. (i) **Delivery of lipids to symbiosome**: Plasma membrane is non-elastic and it is unable to stretch beyond a small threshold (Gavrin et al. 2017). After the endocytosis, rhizobia start dividing inside the plant cytoplasm within the symbiosome, thus requiring membrane expansion. Additionally, rhizobia in IRLC legumes endoreduplicate inside the symbiosome and elongate up to tenfold. Hence, delivery of the membrane material is very crucial for the expansion of the symbiosome membrane. According to the proposed model, the endoplasmic reticulum (ER) and Golgi-derived vesicles fuse with symbiosome and promote symbiosome membrane expansion. Overexpression of MtN5, a lipid transfer protein leads to increased nodule number suggesting that lipid delivery to symbiosome is a rate-limiting process (Pii et al. 2009). (ii) **Delivery of proteins to symbiosome**: Symbiosome-specific protein targeting machinery is indispensable in maintaining its independent organelle-like identity. A dedicated protein trafficking pathway has been depicted which is required for the delivery of small peptides to symbiosomes in IRLC legumes (described in detail in Sect. 18.3.2). Proteins are targeted to (a) symbiosome membrane (b) symbiosome space, and (c) the bacteroids. How this differential protein targeting is achieved is not clear. From the metabolic point of view it can be easily visualized that transporters need to be targeted towards the symbiosomes. Among them sugar transporters (LjSUC and MtSWEET11), a sulphate transporter (LjSST1), an iron induced-citrate transporter (MtMATE67), and an aquaporin Nodulin 26 have been shown to localize to the symbiosomes (Flemetakis et al. 2003; Wienkoop and Saalbach 2003; Krusell et al. 2005; Kryvoruchko et al. 2016; Kryvoruchko et al. 2018). Several metal ions such as molybdenum, iron and dicarboxylic acid-malate certainly need to be delivered inside the symbiosomes, whereas ammonium needs to be transported out. Until now it is not clear which transporter is delivering those. Several vesicular trafficking proteins are further shown to be targeted towards symbiosome (such as vascular SNAREs SYP22 and VT11, ENOD8) (Limpens et al. 2009; Meckfessel et al. 2012). (iii) **Control of defence response during symbiosome development:** Flagellin acts as an elicitor to pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). Flagellar regulon is expressed in bacteroids, suggesting that there is a high potential of triggering PTI while rhizobia are present inside the nodule (Roux et al. 2014). On top of that, a huge number of symbiosomes are present inside a single plant cell in the infection zone. Nonetheless, the defense response is not elicited inside nodules. Then how is plant immunity modulated inside the nodule? A few plant mutants that have fixation +/− phenotype generate a profound defence response. Until now, four plant mutants have been

reported with accumulation of phenolic compounds, which are a sign of defence response. There causative genes are *MtDNF2* (*defective in nitrogen fixation 2*), *MtSYMCRK (Symbiotic cysteine-rich receptor-like kinase)*, *MtRSD* (*Regulator of symbiosome differentiation*), and *MtNAD1* (*nodules with activated defense 1*) (Bourcy et al. 2013; Sinharoy et al. 2013; Berrabah et al. 2014, Wang et al. 2016). Probably these four genes are required to keep plant immunity at check during symbiosome development. Connection between these genes and a downstream signalling pathway that modulates the plant immunity is not clear (Berrabah et al. 2015; Domonkos et al. 2017). RSD is a transcription factor. It is expected that direct targets of RSD control nodule development. It has been shown that RSD represses a vesicular trafficking protein (*MtVAMP721a*) (Sinharoy et al. 2013). Our recent data indicate that several other RSD targets play a major role in controlling plant immunity (unpublished data). The above-mentioned four genes are only reported in *Medicago,* which forms indeterminate nodules. Whether these signalling molecules work similarly in determinate nodules where terminal differentiation of bacteroids does not occur remains an open question. Overall, control of plant immunity in determinate nodules has not been investigated in detail.

18.5.3 Terminal Bacteroid Differentiation

In some species, bacteroids go through a host-induced terminal differentiation and dramatically change their shape (Mergaert et al. 2006). Bacteroid terminal differentiation seems to be linked to high nitrogen fixation rates (Oono et al. 2010). In *Medicago*, bacteroids undergo endo-reduplication up to 24C compared to 1–2C for free-living bacteria (Mergaert et al. 2006). Terminally differentiated *Medicago* bacteroids are very elongated or have a Y shape. Dalbergoid plants induce terminal differentiation in bacteroids, which become either swollen spherically shaped (e.g., *A. indica* and *A. hypogaea*) or elongated (*A. afraspera*). Transcriptome analysis in *M. truncatula* revealed that more than 700 small nodules-specific-cysteine-rich (NCR) genes are induced during symbiosis. The induction of *NCR* genes has been reported in wide range of IRLC legumes and some Aeschynomenoid nodules (Montiel et al. 2017). The role of NCR peptides in mediating terminal bacteroid differentiation remains a process yet to be mechanistically understood. NCR peptides are 60–90 amino acid long defensin-like peptides with conserved cysteine motifs but otherwise are quite diverce. These peptides induce membrane permeability, endoreduplication of the bacteroid genome, and loss of viability in the free-living state (Van de Velde et al. 2010). NCR peptides contain an N-terminal signal sequence that is processed by the ER-localized endopeptidase complex. One of the subunits of the peptidase complex is DNF1 (Wang et al. 2010). Mutation of *dnf1* blocks bacteroid differentiation, and the NCR peptides become trapped in the ER without being delivered to the bacteroids (Van de Velde et al. 2010). Eva Kondorosi's group did phenomenal work in discovering NCR gene families across IRLC legumes. It has shown that NCR247 interacts with bacteroid protein FtsZ to inhibit bacteroid cell division and promote cell elongation (Farkas et al. 2014; Penterman

et al. 2014). Disulfide cross-linkings of cysteine present in the NCR peptide are crucial for its activity (Shabab et al. 2016). Certain *ncr* mutants of *Medicago* (e.g., *dnf4-NCR211* and *dnf7-NCR169*) cannot fix nitrogen (Horvath et al. 2015; Kim et al. 2015). Furthermore, two NCR peptides coded by nitrogen fixation specificity genes (*NFS1* and *NFS2*) also function as negative regulators of strain-specific symbiont persistence (Wang et al. 2017; Yang et al. 2017). The specific role of only a few NCR peptides have been revealed amidst the overall abundance of *NCR* genes expressed in nodules. Further, the absence of the symbiotically essential *NCR*s in most of the other IRLC family members suggest a complex regulation in the evolution of terminal differentiation of bacteroids. Interestingly, *NCR* genes are not expressed in many species with determinate nodules or are even absent in the genome of these legumes, including *Lotus* (Mergaert et al. 2003; Kereszt et al. 2011). Recently we have performed the RNAseq profiling of developmental stages of *A. hypogaea* (peanut, Aeschynomenoid/Dalbergioid) nodules. Interestingly, we were unable to detect any expression of *NCR* genes in the transcriptome (unpublished data). As aforementioned, *Arachis* forms swollen spherical symbiosomes, which suggests they undergo terminal bacteroid differentiation (Oono et al. 2010). Hence, it is interesting to note that the convergent evolution of NCR-mediated bacteroid terminal differentiation that takes place in the IRLC clade and certain aeschynomenoid legumes has not followed the same evolutionary trajectory in terms of molecuar mechanisms in *Arachis*.

18.6 Concluding Remarks

In summary, let us revisit the question asked by Ann M. Hirsch, Thomas A. Larue, and Jeff Doyle about 20 years ago in their review 'Is the legume nodule a modified root or stem or an organ *sui generis?*' (Hirsch et al. 1997). With our current understanding of the nodule nature and evolution, the answer is still not clear. However, with the new information available we can reasonably assume that the nodule identity is probably a cocktail of all the three developmental pathways. Future studies should focus on alternative symbiosis mechanisms. Such studies will not only help us better understand the nature of this unique economically relevant process, but also will show the direction to transfer this valuable trait to non-nitrogen fixing crops.

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CRISPR Applications in Plant Genetic Engineering and Biotechnology

19

Vivek Srivastava

Abstract

Genetically modified (GM) crops were first introduced for commercial use in 1996 and as of 2017 were grown on 190 million hectares in 26 countries. Even with the potential these crops believed for worldwide food security, GM crops lately were linked with uncorroborated health concerns, environmental safety, ethical issues and restrictions in European countries.

Over the course of recent years, through improvements in the plant research, new methods have been developed allowing for unambiguous mutations with precision. This includes technologies like mega nucleases, TALENs and zinc finger nucleases (ZFN). These genetic editing techniques are complex, difficult to manipulate constructs and costly. The approval of genetic editing has potential for inherent improvement of crops, improved disease tolerance, improved food, energy, and lint (fibre) security across world. Most importantly, the technology has potential of creating new varieties of crop, tastier vegetables and fibre full grains.

Advances in CRISPR/Cas9 radically cut the cost of performing genetic editing trials, easier implementations and permitted for prolonged use of the technology in plant sciences. We describe here assessment on development, use and trials of CRISPR/Cas9 technology and its implication in crops basic research and crop genetic improvement.

Keywords

CRISPR · Cas · Genetic manipulation · Homologous recombination · NHEJ · Heritable genetic mutation · EFSA · USDA

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19.1 Introduction

The improvements in genetic manipulation methodologies promise a tangible benefits and innovation in genetic engineering. Amid the past years, some innovative gene editing systems have been established, consisting of zinc finger nucleases (ZFNs), oligonucleotide directed mutagenesis (ODM), transcription activator like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9). Amongst these strategies, CRISPR/Cas9, have substantiated itself as effective and reliable tool for genetic editing. CRISPR has been applied in the dairy industry since long, (*Streptococcus thermophilus* genome contains odd chunks of repeated DNA sequences) and over many years gained significance by virtue of its ability to exactly target selected sequences in genomes. CRISPR repeat sequences were originally observed in *Escherichia coli* and *Haloferax mediterranei* (an Archaea) as an adaptive microbial immune systems against bacteria or virus (Ishino et al. 1987; Mojica et al. 1993). CRISPR/Cas type IIA system present in the bacterium *Streptococcus pyogenes* is the most standard CRISPR technology that depends on the use of complementarity of the synthetic guide RNA (sgRNA) to a precise genome sequence and the nonspecific Cas9 endonuclease. By creating nucleotide variants of the 20 base pair target sequence in the gRNA and its capacity to generate mutations and insertions in plants genome, targeting single or multiple locations is conceivable. For this reason, CRISPR/Cas9 has recognised to be incredibly significant for high throughput genome engineering. CRISPR procedure depends on the use of sequence defined or specific endonucleases (SSNs) to create a break in double strands of DNA (DSB) at distinct genetic location, which are then repaired by pathways, mainly: nonhomologous end joining (NHEJ) (Gorbunova and Levy 1997) and homology directed repair (HDR or HR) (Liang et al. 1998). DSB's repaired by NHEJ mechanism cannot be predicted and can generate frame shift mutations that frequently creates genetic knockouts (KO). These KO germ lines allows generation of variant alleles which are valuable for functional and reverse genetic studies with relevance in agriculture.

The knowledge around CRISPR/Cas is progressing and the reports are extensively reviewed on safety and applications. Here is a brief summary on the development of CRISPR technology, their applications and challenges.

19.2 CRISPR/Cas9 Workflow in Plants

Introduction of genetic editing tools permits exact manipulation of plant genomes thereby superseding established methods of random mutagenesis as Ethyl methanesulfonate (EMS) and gamma radiation sequences, as a result reducing laborious screening of enormous populations to detect mutants (Sikora et al. 2011). CRISPR can be used to create KO (allele variant), activate or supress target genes of undesired or desired traits in plants using standard experimental design outlines

developed in any other model organisms. Still, plant specific modifications to commonly used CRISPR plasmids are necessary to utilize the system. CRISPR/Cas system consists of genes ciphering ((CRISPR RNA) crRNA with (transactivating crRNA) tracrRNA), which makes for gRNA, a nonspecific endonuclease Cas9 from *Streptococcus pyogenes* (SpCas9) (Makarova et al. 2015) and perceives NGG type Protospacer adjacent motif (PAM) at the 3′ end of target (Mojica et al. 2009). Also advance variants, novel RNA guided nucleases and orthologous of Cas9 were recognized and extended the diversity of PAM sequences increasing specificity while editing (Kleinstiver et al. 2015; Steinert et al. 2015). Choice of PAM sequences is determined by species source of the applied Cas9 protein. Cpf1 is recently identified class II RNA guided endonuclease, type V CRISPRCas system to mediate targeted genome modification in different cell types (Zetsche et al. 2015; Kim et al. 2016). Cpf1 recognizes the thymidine rich PAM and makes staggered cuts distal to the PAM site (Fonfara et al. 2016; Strohkendl et al. 2018) the mechanism is different from Cas9 which generates blunt end DSB's.

In plants, efficient CRISPR–Cas9based genetic editing usually comprise four steps (Fig. 19.1) (Schiml et al. 2016; Yin et al. 2017).

First, designing gRNA sequence for selected genome region. It includes target sequence of 20 bp followed by PAM (NGG).

Second, assembling codon optimized Cas9/sgRNA construct with plant RNA polymerase III promoter (AtU6 or TaU6 or OsU6 or OsU3). The efficiency of sgRNA is confirmed in protoplasts prior its use in genetic editing.

Third step, to edit, transformation of CRISPRCas9 into plant tissues, via *Agrobacterium* mediated transformation or particle bombardment, for stable integration of Cas9 and sgRNA cassette into genome.

Fourth Step, mutant or transformed (regenerated) plants with the anticipated changes are recognised by polymerase chain reaction (PCR) genotyping or surveyor assay (such as Mismatch Cleavage, Heteroduplex mobility, High resolution melting etc.) and confirmed by sequencing.

Genetic editing methodologies in plants using combination of Cas9ciphering mRNA and guide RNA or preassembled ribonucleoproteins (RNPs) develop non transgenic, regulatory free plants with low transient expression (Fig. 19.2). While

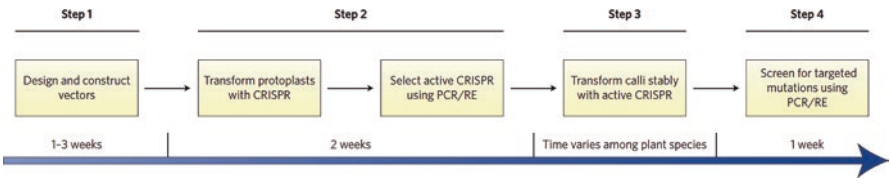

Fig. 19.1 General Procedure of plant genome editing using CRISPR-Cas9. Plant genome editing can be typically be divided into four continuous steps, and the estimated time needed for each step is indicated. PCR/RE, polymerase chain reaction/restriction enzyme digestion. (Adapted from 10.1038/nplants.2017.107)

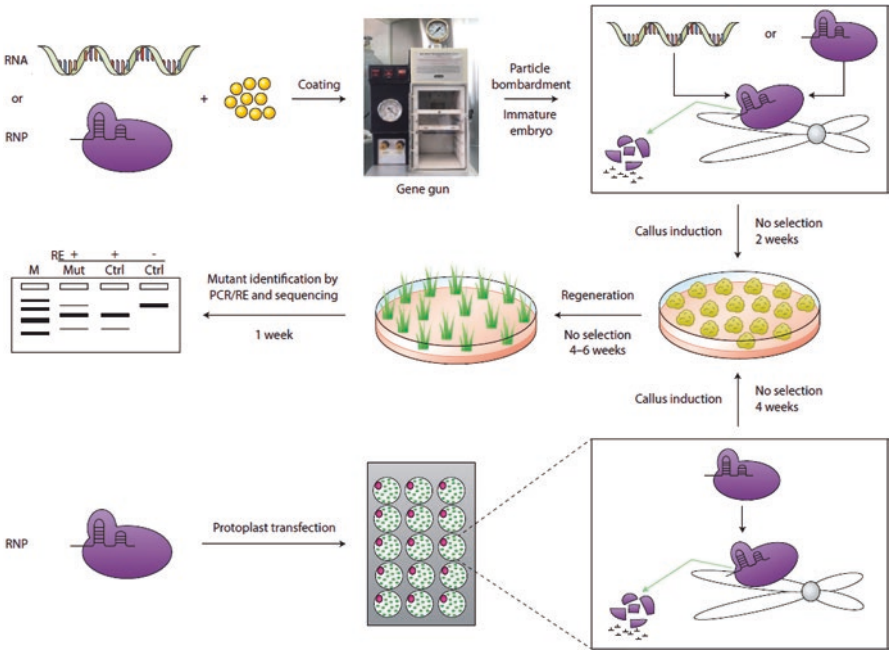

Fig. 19.2 DNA free gneome editing with CRISPR-Cas9 RNAs and RNP in plants. The CRISPR–Cas9 RNAs (in vitro synthesized Cas9 and sgRNA transcripts) or pre-assembled CRISPR–Cas9 RNP can be delivered into immature embryos via particle bombardment. Alternatively, pre-assembled CRISPR–Cas9 RNP can be transfected into plant protoplasts. Bombarded/transfected cells are induced to form calli, from which seedlings are regenerated under the selection-free conditions. Regenerated plants are screened for mutation via PCR/RE assay and sequencing. The estimated times needed are indicated for most steps. Delivering CRISPR–Cas9 reagents via RNP limits their temporal activity, thereby improving their precision. RE, restriction enzyme;M, DNA marker; mut, mutant; ctrl, control. (Adapted from 10.1038/nplants.2017.107)

harbouring insertions and/or deletions (random point mutations or indels) at the intended site, plants regenerated with CRISPR–Cas9 may comprise undesirable insertions at both on and off target sites (Feng et al. 2013; Shan et al. 2013). These mutants are also frequently considered to be GMOs and might be subject to regulatory studies in few nations.

There is a need to adjust in silico design for information gathering, approval and methodical investigation of sgRNA efficiencies. The CRISPR Cas9 can henceforth be termed as a method with extraordinary potential that is easy to use because of its straightforwardness, effectiveness, and wide abilities.

19.3 CRISPR/Cas9 Applications in Plant Genetic Editing

In series of comprehensive articles and studies, it was demonstrated that how CRISPR/Cas9 system has been extensively applied to perform (Khatodia et al. 2016; Soyars et al. 2018)

(a) Multiplexing genetic engineering (Li et al. 2013a, b)
(b) Genomic engineering through management of transcription (Zalatan et al. 2015)
(c) Genomic engineering through DNA repair (Jiang et al. 2013)
(d) Genomic engineering through heritable genetic mutations (Schaeffer and Nakata 2015)

An overview on achievement of gene editing methods the scientific significance, and what research needs to be done is discussed in details.

19.3.1 Multiplexing Genetic Engineering

CRISPRCas9 is used to achieve multiplex genetic editing (MGE) by flexibility to add more gRNAs on the similar or extra target DNA (TDNA) construct with possibility to utilize similar or many different promoters (Xing et al. 2014; Wang et al. 2015a, b). MGE have been successfully applied in both monocots and dicots plants like wheat, maize and arabidopsis (Xing et al. 2014; Ma et al. 2016).

A few novel methodologies have been created for MGE. The Golden Gate cloning or Gibson assembly (Engler et al. 2009) where, the resultant vector contains a codon optimised Cas9 and numerous gRNAs transcribed by U6 promoters. Codon optimised Cas9 endonuclease (high %GC) and plasmids are designed for both monocot and dicot plants, with a choice of either Basta (Glufosinate Ammonium) or hygromycin selection. Albeit challenging, this methodology took into account simultaneous altering of different target sequences in genome. Controlling exact processing of tRNAprecursors, various (6–12) sgRNAs with exact origin sequence could be handled in vivo from a single transcript by endogenous tRNA processing machinery (Zhang et al. 2016; Cermak et al. 2017). Extension of second strategy demonstrating assembly isocaudamer procedure with compatible endonucleases (*BsaI*) was created and validated utilizing maize protoplasts and arabidopsis transgenic lines showing high coherence and specificity for introducing mutations in multiple genes. PCR and sequence analysis confirmed heritable changes in later generations (Wang et al. 2015a, b). Gao et al. 2018, used MGE-CRISPR/Cas9 to edit the *SPL9*gene, giving acumens into prospects in future alfalfa breeding.

Furthermore, scientists attempted to coordinate MGE for controlling plant infections (Zaidi et al. 2016). To achieve this, Tobacco rattle virus (TRV) was used to convey sgRNAs to transgenic *Nicotiana benthamiana* that stably overexpressed Cas9 bypassing requirement for transformation, enable multiplexing, improving editing efficacies and applicability of knowledge in various plant species (Ali et al. 2015).

Presence of CRISPR/Cas9 tolerant and PAM free areas are likewise spread in genomes, where this methodology is intractable. Zhao et al. (2017) depicted CRISPR/Cas9 assisted gRNA free, single step (CAGO) system which uses an all inclusive N20 sequence addition in genome by an intra chromosomal HR event to finish the editing procedure with no off target impacts. When applied to MGE, CAGO achieved 100% efficiency for single locus. Moreover, CAGO was used to edit huge DNA regions (up to 100kbp) with 75% efficiency. These findings opened novel prospects in creating multiple genetic changes by stacking various qualities in an elite variety background, capably refining complex agronomic attributes (Yin et al. 2017).

19.3.2 Genomic Engineering Through Management of Transcription

RNA guided Cas9 genetic editing can also be repurposed by manipulating transcription. Manipulating the temporal expression of specific genes during plant development using artificial transcription factors is valuable for research pertaining crop improvement. Jinek et al. (2012) deactivatedCas9 (dCas9) nuclease function by introducing point mutations in its two catalytic domains, *RuvC* (D10A) and *HNH* domain (H840A) (N863A) (Nishimasu et al. 2014), converting the CRISPR/Cas system from a genetic editing tool to a specifically targeted genome regulation tool as epigenetic effectors (Fig. 19.4). Coupling dCas9 with gRNAs targeting the promoter sequence of a gene, the complex becomes a potent tool for the transcriptional repression of genes and known as CRISPR interference (CRISPRi). The binding of gRNA in upstream region (50 bp and +300 bp) of transcription start site (TSS) were recognised with maximum silencing by CRISPRi (Gilbert et al. 2014).

ThedCas9/sgRNA impact can be amplified through coupling of a repressor protein (Kruppel associated box: KRAB domain) or a coupling of a transactivation domain (VP64) causing repression or activation of transcription of gene in study (Konermann et al. 2015). A study by Piatek et al. 2015 achieved transcriptional activation on *Bs3::uidA* targets in plant cells by fusion of C terminal of the codon optimized dCas9 with EDLL domain of ERF/EREBP family of plant transcription promoter elements and the TAL activation domain. Simultaneously in same study transcriptional repression of endogenous gene was observed for dCas9: SRDX fusion construct. The repression or activation of transcription was interpreted in inoculated *Nicotiana benthamiana* leaves through estimation of expression levels of transient GUS gene/endogenous phytoene desaturase gene (*PDS*). Lowder et al. (2017) demonstrated in Arabidopsis that concurrent fusion of VP64 to dCas9 and a modified guide RNA scaffold gRNA2.0 (CRISPR Act 2.0; different from standard CRISPR) yielded even stronger transcriptional activation compared to dCas9-VP64 alone leading to conclusions that some endogenous genes are more prone to transcriptional activation than others and tightly regulated genes resulted in strong transcriptional repression with CRISPRAct2.0 (Lowder et al. 2017; Lowder et al. 2018).

Polstein and Gersbach (2015) engineered light activated CRISPR effector (LACE) system where by using blue light as inducer, transcription of internal

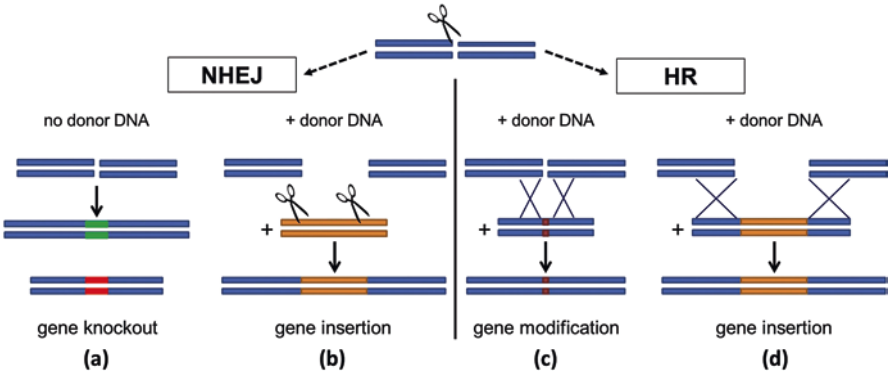

Fig. 19.3 Genome editing with site-specific nucleases. Double-strand breaks induced by a nuclease at a specific site can be repaired either by non-homologous end joining (NHEJ) or homologous recombination (HR). (**a**) Repair by NHEJ usually results in the insertion (green) or deletion (red) of random base pairs, causing gene knockout by disruption. (**b**) If a donor DNA is available, which is simultaneously cut by the same nuclease leaving compatible overhangs, gene insertion by NHEJ can also be achieved. (**c**) HR with a donor DNA template can be exploited to modify a gene by introducing precise nucleotide substitutions or (**d**) to achieve gene insertion. (Adapted from 10.1016/j.biotechadv.2014.12.006)

control or housekeeping or reference genes was achieved by coupling the light driven heterodimerizing proteins *CRY2* and *CIB1* to a transactivation domain and dCas9. The multipurpose LACE system can be directed to novel DNA sequences making dynamic regulation of internal control genes plausible.

Very recently, nuclease Cas13a has been identified for post transcriptional regulation of gene expression and combating viral infections in plants (Wolter and Puchta 2018). Intriguing applications are with nuclease dCas13 empowering to visualize single strand RNA in vivo, and to edit at specific bases of RNA molecules by deamination or to modify them by conjugation. This application will have unique potential for crops.

Investigation of features like low cytotoxicity, inconsistency of function among organisms and test conditions due to dependence on cytoplasmic components for silencing, and fewer off targets in plants will enhance future use of the dCas9/sgRNA repression or activation system. Overall, it was demonstrated that synthetic dCas9 transcriptional regulators in plants could enable functional analysis of genes in diverse nutritional and environmental situations. These studies open exciting possibilities to address important questions on gene regulation in agricultural biotechnology.

19.3.3 Genomic Engineering Through DNA Repair

DSB repair mechanism involves NHEJ or HR that produces genetic alterations, conditional gene knockouts and gene insertions (Fig. 19.3) (Ma et al. 2015). NHEJ

is the common and fastest DSB repair tool in somatic cells. The error prone NHEJ generates arbitrary insertions or deletions in the coding sequences prompting change in reading frames, consequently creating gene knockouts. For e.g., powdery mildew resistant wheat line produced by knocking out three redundant *MLO* genes.

For development of varied range of agriculturally significant traits, HR mediated genetic editing is desired but the method is limited by low efficiency and sluggish recombination. HR pathway inserts exogenous DNA into genome utilizing possible homologous matrices generated from homologous sequences. A broader effort is required for improving HR efficiency by using*SDN2* and *SDN3* (HR boosters) or NHEJ inhibitors, which are currently being explored in mammalian cells (Podevin et al. 2013).

An enhancement of the DSB induced gene targeting, called *In planta* gene targeting approach (IPGT) (Fauser et al. 2012), independent of transformation and regeneration processes, consists of coordinated introduction of DSB to the target region and excision of the repair template (donor) from the plant DNA where it was previously introduced. Researchers have endeavoured using 18 bp mega nuclease *ISceI* restriction site that permits gene targeting in arabidopsis without depending on high transformational rates (Fauser et al. 2014). Using nonspecific endonuclease Cas9 decreased the number of T DNA from three (donor sequence, *ISceI* target sequence and *ISceI* expression system) to single (Schiml et al. 2014) achieving targeted flexibility and site specific insertion in *Arabidopsis* genome.

Using *Arabidopsis thaliana*, alternative method was developed by packaging Cas9 and gene targeting replicons in geminiviruses (Homology template with ~600 to 800 bp arms on both ends), flanked by LIRs (Long intergenic regions), SIR (Short Intergenic regions), and a replicase (*Rep/RepA*) for effective delivery. Since Gemini viruses don't integrate in host genomes, plants gene(s) edited by DNA replicons might be devoid of gene targeting reagents. The viral replication initiation protein (Rep) initiates circled and rolling circle replication of replicons at LIR, inter genetic regions creating single strand nicks. Following rolling circle replication these nicks are converted to DSBs whereby strongly enhancing gene targeting events successfully tested in tomato (Baltes et al. 2014). Analogous to the Geminivirus vector, Cabbage Leaf Curl virus was used for gRNA delivery in *Nicotiana Benthamiana* (Yin et al. 2015). MGE through biolistic transformation and regeneration of immature embryos, copy number variations of the repair sequence together with DNA cleavage efficiently promotes gene targeting via HR in tomato (Cermak et al. 2015) soybean (Li et al. 2015) maize (Svitashev et al. 2016) Potato (Butler et al. 2016) rice (Sun et al. 2016) and hexapolid wheat (GilHumanes et al. 2017). Successful biallelic editing with high efficiency targeting multiple (5) genes in immature maize embryos was achieved by optimized Cas9 and gRNA with relatively few transformations generating just sufficient plants containing desired mutations in both alleles (Weeks et al. 2016). Hayut et al. (2017) demonstrated genetic cross over in heterozygote tomato plants stably expressing Cas9 and gRNA independently displayed exact rearrangement of homologous chromosomes in somatic cells by HR. This experiment derived utility of CRISPR at distant parts of a chromosome by targeted crossover.

The low frequency of HR remains the major obstacle but recent progress to transform and revive plants in culture, greatly increasing the efficiency in maize

inbred lines by upregulation of Baby boom (*Bbm*) and Wuschel2 (*Wus2*) also observed in sorghum (*Sorghum bicolor*), sugarcane (*Saccharumofficinarum*), and rice (*Oryzasativa* ssp. *indica*) (Lowe et al. 2016). *Bbm* and *Wus2*expressioncompensate the low efficiency observed in monocots (intensive labour and greenhouse) to resource immature embryos for transformation. Overexpression of Phosphomannose isomerase (*PMI*) and Ovule development protein2 (*ODP2*) also enthused transformation and regeneration in maize (Svitashev et al. 2016). Ectopic expression of *WIND 1*, transcription factor, in *Brassica napus*, can also improve regeneration capability (Iwase et al. 2015). Essentially, not every transgenes transformed into plant cells achieves a stable integration. In rare cases, an unintegrated T DNA (with stable marker gene) and a stably integrated gene of interest may coexist in some of cells. Interestingly, with a small selection phase, unintegrated transgenes can still be expressed, providing another method of plant genetic editing. The presence of T DNA also allows for marker free selection of transgenes in subsequent generations (Yau and Stewart 2013).

For efficient visual detection of HR events and testing mutagenesis efficiencies, another strategy consists of using target specific sgRNA(s) and another sgRNA targeting an internal control or endogenous gene where mutation leads to prompt selectable phenotype. *Glabrous1 (GL1)* is required for the formation of trichomes in arabidopsis (Hahn et al. 2017), the idea being to detect heterozygous mutations using a reverse approach by HR based restoring the *GL1* gene via Cas9 in the seedlings presenting the phenotype.

NHEJ and HR assists cells to accomplish repair of DSBs and other intricate DNA damages. It is vital to do further research on NHEJ or HR editing pathways and their applications in plant biotechnology.

19.3.4 Genomic Engineering Through Heritable Genetic Mutations (HGM)

CRISPR/Cas9 advancement and optimization of technology, improved its consistency and efficacy to create heritable genetic changes in plants.

Van Eck group from Cold Spring Harbor Laboratory demonstrated Cas9 mediated heritable mutations in tomato by deletion of *SIAGO7*gene (Brooks et al. 2014). Only 50% of transformants displayed homozygous mutation early in development. On sequence analysis revealed the deletion of gene confirming smaller inherited mutations, phenomena similar to confirmation through pollination of wild type plants by mutants. Heterozygous plants (lacking Cas9 transgene) with one wild type and one mutant allele could be identified, thus confirming heredity of the mutations. Pan et al. 2016 targeted two genes *SlPDS* and *SlPIF4* through an *Agrobacterium* mediated transformation achieving 84% mutation frequency in all tried targets in the T_0 transgenic plants, with clear albino phenotypes for the *psd* mutants. High frequencies of homozygous and biallelic mutants were also noticed in T_0 plants. Frequently detected mutations were 1to3 nucleotide deleted, followed by 1 bp

inserts. The target mutations from T_0 lines stably integrated into T_1 and T_2 generations, without new modifications.

Zhou et al. (2014) used rice codon optimized Cas9 under a maize ubiquitin promoter (U6) stably controlling activity of a complex produced by use of two differentiated sgRNA with 48 and 85 nt RNA tracr tails introducing HGM. Stable transformed rice plants were tested and it was learned that sgRNA (48 nt) did not produce evident mutation. In spite, sgRNA (85 nt) indicated higher efficiency mutations by initiating biallelic mutations in all the transformants further prompting creation of transgene free progeny (by classical genetic segregation) in second generation. In other studies too stable inheritance of Cas9interceded mutations were accounted, when Feng et al. 2014 used the human codon optimized Cas9 (hCas9) under the control of two CaMV 35S promoters and 12 different sgRNAs driven by the AtU6 26 promoter to target endogenous sites in arabidopsis. They reported that plants bearing any mutation whether chimeric, heterozygous, biallelic, or homozygous were steadily inherited at higher recurrence for next T1, T2 and T3 generations.

Xing et al. (2014), using a set of different constructs (maize codon optimised zCas9 and TaU3 promoter for the sgRNA), displayed high efficiency focussed mutagenesis in both maize protoplasts and transgenic plants. Heritable mutations in progressive generations were further confirmed by PCR and sequencing. Sequencing of the transformants also highlighted small insertions of 1 bp considered most prevailing for genetic alterations.

In *Hordeum vulgare*, two copies of *HvPM19* gene were targeted and mutations were steadily transmitted and recognised in the first generation (23% and 10% lines, respectively) (Lawrenson et al. 2015). CRISPR focusing *BolC.GA4.a*from brassica, instigated induced mutations in 10% of first generation selected plants. Additionally, a phenotypic screen, distinguished T_0 plants with the expected dwarf phenotype to be associated with KO of target gene. In both barley and brassica, stable Cas9 induced mutations were transferred to T_2 plants independently of the T DNA construct. However, off target activity was observed in both species, despite the presence of at least one mismatch between the single guide RNA and the nontarget gene sequences.

Very recently Durr et al. 2018 increased the heritability frequency using CRISPR/Cas9 deletions in arabidopsis carbon/nitrogen insensitive 1 (*CNI1*) a plant specific ubiquitin ligase critical for carbon and nitrogen homeostasis, defence response and leaf senescence.

The above results in tomato, arabidopsis, rice, maize, soybean and so forth approved this enhanced CRISPR/Cas9 methodology can provide a quick, productive and cost effective tool to contrive heritable chromosomal deletions.

19.4 Concerns Regarding 'Off Target' Effect of CRISPRCas9

CRISPR/Cas9 genetic editing can result in changes at non target genetic locations. Off target effects, in which Cas9 cleaves genomic DNA sites that are not exact complements of sgRNA, is one noteworthy downside's of the CRISPR–Cas9 and its detection can be moderate and costly (Zhang et al. 2018). Such 'off target' impacts may meddle with chromosomal stability or loss of functional gene activity causing obscure, non quantifiable cellular signalling or physiological impacts (Guilinger et al. 2014).

The challenge is to report recognition of off target sites in an extensive and sensitive way. Whole genome sequencing (WGS) of T_1 & T_2 plant harbouring GAIsgRNA1 in arabidopsis and rice mutants has demonstrated that the CRISPR/Cas9 system is profoundly explicit in plants (Feng et al. 2014). Whole genome sequencing of 178 off target sites indicated no conspicuous Cas9 induced off target events (Peterson et al. 2016). Sequencing of bioinformatically recognised putative off target sites demonstrated no perceivable events in arabidopsis, tobacco, wheat, rice and sweet orange (Upadhaya et al. 2013; Li et al. 2013a, b; Jia and Wang 2014a, b).

An examination by Jacobs et al. 2015 covering the use of Cas9 to soybean detailed 13% off target recurrence. In this study, two paralogues of DDM1 were targeted and thusly the high sequence similarity is probably causing off target activity. Despite the fact that WGS is perfect for off target mutation assays, a surprising expense goes into it, making it for all intents and purposes difficult to assess a large number of plants and sgRNA variants to determine Cas9 specificity overlooking low recurrence off targets. Because of exceptionally slender number of sgRNA and plants from limited generations, the information cannot preclude the probability of low frequency off target events induced by same sgRNA and high frequency off target events incited by other sgRNA variants (Feng et al. 2014).

It was detected that off target mutations in plants are infrequent and low frequency (Wolt et al. 2016). A study involving use of CRISPR/Cas9 system in rice (*Oryzasativa*) showed deletion events which might be triggered due to NHEJ repair of 5′ overhangs resulting from Cas9induced DNA breaks (Zhang et al. 2014). Targeted sequencing of 13 putative off targets sites for 3 sgRNA in rice found only a solitary mismatch distal to PAM in off target location. Haeussler et al. (2016), reported unexpected observations of high frequency off target mutations induced by a (sgRNA) sgRETC2 with a high specificity score of 94.

To alleviate off target activity of CRISPR/Cas9, a lot of methodologies have been applied. An optimal or transient nuclease expression by controlled release of modified endonucleases from expression plasmids. Intended to decrease "off target effects" includes using a Cas9 nickase variant and trimming gRNA sequences used for targeting which in turn effects on target efficiency (Fig. 19.4) (Casini et al. 2018). For easy packaging, delivery, high fidelity, efforts are also made to decrease the size of Cas9 nucleases (Friedland et al. 2015). Additionally, viral vectors with nonintegrating features are most favoured for minimizing 'off target' actions (Holkers et al. 2014). When preassembled RNP's were used, off target mutations

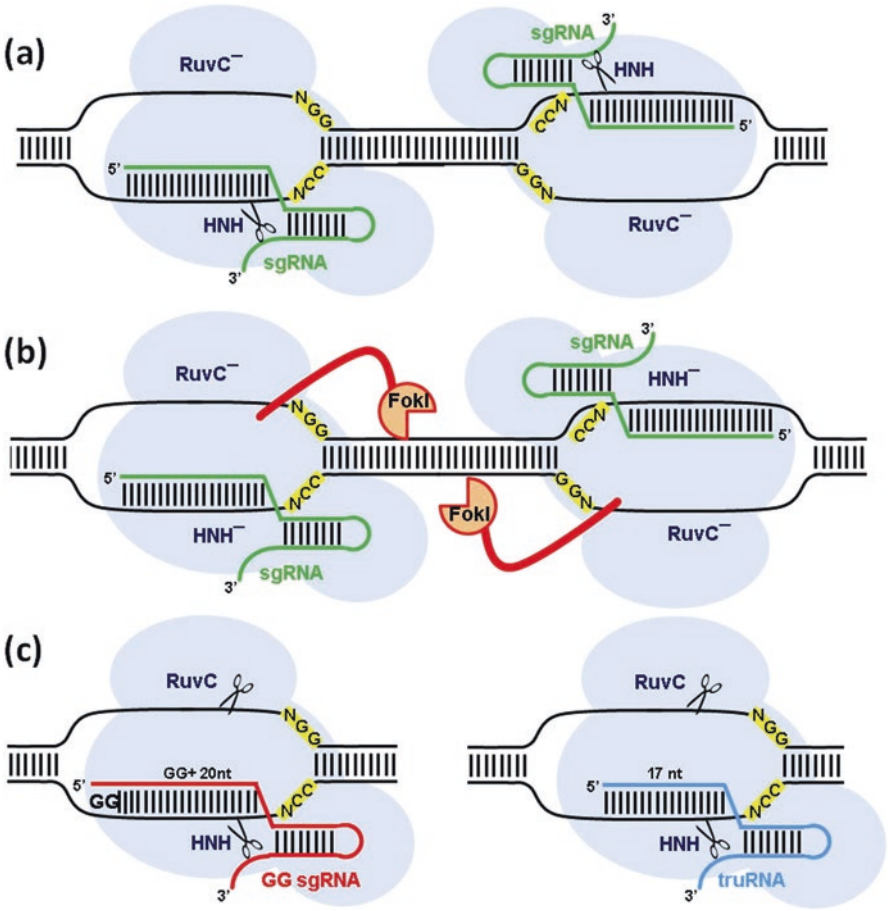

Fig. 19.4 Strategies to increase CRISPR/Cas9 target specificity. The most important strategy to avoid off-target effects is the design of a specific gRNA by checking for the presence of homologous sequences in the genome. Further strategies can then be employed to reduce the risk of off-target cleavage further. (**a**) A pair of offset Cas9 nickases. The D10A mutation inactivates the RuvC endonuclease domain so that Cas9 can cleave only the DNA strand complementary to the gRNA. The simultaneous use of two Cas9 nickases binding to sequences on opposite DNA strands generates a staggered DSB with overhangs. (**b**) Cas9-FokI fusion proteins. A catalytically inactive Cas9 variant, carrying a mutation in both endonuclease domains (RuvC$^-$ HNH$^-$), can be fused to the FokI nuclease domain. DNA cleavage by FokI is dependent on dimerization and the enzyme must bind two precisely disposed half-sites on the genome, greatly reducing the number of possible off-target sequences. (**c**) Altering the length of the gRNA. Both extending the gRNA by adding two guanidine residues at the 5′ end (left), or shortening it to a truncated gRNA (truRNA) of only 17 nt (right) can reduce off-target effects. (Adapted from 10.1016/j.biotechadv.2014.12.006)

were barely detected. Direct delivery of mRNA (instead of delivering plasmid coding for enzymes) too minimized off target events reported in arabidopsis, tobacco, lettuce, rice (Woo et al. 2015), wheat (Liang et al. 2017), maize (Svitashev et al. 2016), petunia (Subburaj et al. 2016).

For each endogenous gene locus, adequacy of DNA cleavage (both target and off target) depends on the nuclease activity as well as on the ease of access of a target site and affinity of the DNA binding domain (e.g., TAL effector domains and gRNA) to the target sequence. For proficiently binding to target sites, Guilinger et al. (2014) and Tsai et al. (2014) fused a Fok1 nuclease domain to dCas9creating a dCas9Fok1 protein needed to form a dimer for successful gene targeting using two sgRNA, whose recognition sites are sequestered by 13–18 bp permitting Fok1 nuclease dimerization and nuclease activity. This again decreased off target impacts because of the exceedingly small recurrence of two off target sites simultaneousness at separation of 13–18 bp. Kleinstiver et al. (2016) devised a high fidelity version of the *Streptococcus pyogenes*Cas9 (SpCas9HF1) to reduce nonspecific DNA contacts by four additional mutations and has enormously reduced the off target effects to basically nondetectable levels when tried in human cells.

Other basic methodologies is to utilize software tools for anticipating off target sites while performing typical sgRNA design and then selecting those sgRNAs that have the least tricky off target sites or the lowest number of them. Some of the general tools that gives off target predictions are CRISPRscan (MorenoMateos et al. 2015), CCTop (Stemmer et al. 2015), and Cas9 Online Designer (Guo et al. 2015). These programs have diverse settings for off target recognition permitting up to few confounds in the entire target site (most of the tools) or vary initiating between the most essential 12 nucleotides nearest to the PAM (CCTop) and the rest of the target sequence. CCTop provides one of the most detailed and informative off target site overviews. In addition there are also more specialized programs, such as CasOFFinder (Bae et al. 2014), CasOT (Xiao et al. 2014) and GTScan (O'Brien et al. 2014) that permits more generalized searches for off target sites, but their output may often need additional computational processing and interpretation. Several unbiased approaches, such as BLESS, GUIDEseq, HTGTS and Digenomeseq, have been developed to recognize off target changes in human cells, and these approaches should be adjusted to plants.

19.5 Agricultural Applications of CRISPRCas9

CRISPR has abundant agricultural uses like improvements to crop yields, providing resistance to diseases, tolerance to abiotic stress, as well as advances beneficial to the population as output characteristics and dietary content (Table 19.1). DuPont, Monsanto, and BASF have already licensed the CRISPR tool for use in new crop and seed development.

Table 19.1 List of targeted genes via CRISPR/Cas9 system in different plant species to improve yields and offer resistance to diseases

Crop	Target gene(s)	Results	References
Arabidopsis thaliana	*AtPDS3*	Feasibility and efficacy of CRISPR	Li et al. (2013a, b)
	AtFLS2	Feasibility and efficacy of CRISPR	
	AtADH	Feasibility and efficacy of CRISPR	
	AtFT	Feasibility and efficacy of CRISPR	
	AtRACK1b & AtRACK1c	Feasibility and efficacy of CRISPR	
	TRY, CPC, ETC2	Defense against pest	Xing et al. (2014)
Brassica oleracea	*BolC.GA4*	Dwarf phenotype	Lawrenson et al. (2015)
Camelina sativa	*FAD2*	Seed Oil composition	Jiang et al. (2017)
Cassava	*elF4E (nCBP-1 & nCBP-2)*	Resistance to brown streak disease	Gomez et al. (2018)
Cucumis sativus	*elF4E*	Resistance to Cucumber vein yellowing virus	Chandrasekaran et al. (2016)
Glycine max	*ALS1*	Resistance to sulfonylurea herbicides	Li et al. (2015)
	DD43 region	Resistance to hygromycin	Li et al. (2015)
Gossypium hirsutum	*CLCuD IR & Rep regions*	Resistance to cotton leaf curl disease	Rahman et al. (2017)
Hordeum vulgare	*HvPM19 (ABA-induced)*	Grain Dormancy	Lawrenson et al. (2015)
Lotus japonicus	*SNF*	Symbiotic nitrogen fixation	Wang et al. (2016a, b)
Nicotiana benthamiana	*NtPDS*	Feasibility and efficacy of CRISPR	Nekrasov et al. (2013)
	NtPCNA	Feasibility and efficacy of CRISPR	Ali et al. (2015)
	NtPDR6	Feasibility and efficacy of CRISPR	Nekrasov et al. (2013)
	BeYDV	Resistance to Bean yellow dwarf virus	Baltes et al. (2014)
	TYLCV-IR	Resistance to tomato yellow leaf curl virus	Ali et al. (2015)

(continued)

Table 19.1 (continued)

Crop	Target gene(s)	Results	References
Oryzae sativa	*OsSWEET11*	Resistance to bacterial blight (Xanthomonas oryzae)	Zhou et al. (2014)
	OsSWEET14	Resistance to bacterial blight (Xanthomonas oryzae)	
	OsERF922	Resistance to rice blast disease	Wang et al. (2016a, b)
	OsWaxy	Glutinous rice	Ma et al. (2015)
	Gn1a, DEP1, GS3, IPA1	Grain number per panicle; Plant Height; Seed size	Li et al. (2016)
	ALS1	Resistance to sulfonylurea herbicides	Sun et al. (2016)
Solanum lycopersicum	*ANT1*	Ecotopic pigment accumulation	Cermak et al. (2015)
	SIAGO7	Wiry Leaves	Brooks et al. (2014)
Solanum tuberosum	*GBSS*	High amylopectin	Andersson et al. (2017)
Triticum aestivum	*TaMLO-A1, TaMLO-B1, TaMLO-D1*	Resistance to Powdery Mildew	Wang et al. (2014)
	TaDREB2 & TaERF3	Abiotic stress response	Kim et al. (2018)
Zea mays	*LIG1*	Liguleless 1	Svitashev et al. (2016)
	Ms26 & Ms45	Male sterile tassel	Svitashev et al. (2016)
	ALS	Resistance to sulfonylurea herbicides	Svitashev et al. (2016)

19.5.1 CRISPR/Cas9 in Vegetable Crops

Owing high efficacy and ease, CRISPR/Cas9 has been applied to diverse crops for trait improvements. To explore the gRNA/Cas9 technology various crops were engineered as *Arabidopsis thaliana* (Hyun et al. 2015) *Nicotiana benthamiana* (Gao et al. 2015) and *Oryzasativa* (Song et al. 2016; Yang et al. 2018).

Majority applications of CRISPR are performed in tomato targeting 18 diverse genes independently. Primarily CRISPR mediated genetic targeting in tomato was done on argonaute7 (*SlAGO7*) gene involved in leaf development (Brooks et al. 2014) resulting a needle like or wiry leaves, leading to easy identification of edited

plants. Later, genes regulating plant growth and developmental like short root (*SHR*; root development; Ron et al. 2014), bladeonpetiole (*SlBOP*; inflorescence; Xu et al. 2016) and selfpruning5G (*SP5G*; repressor of flowering) and self pruning (*SP*; plant development; Soyk et al. 2017) were edited to establish their functional purpose in tomato. These mutations leads to a quick burst of flower production and early yields than conventional breeding programs. The standard ripening inhibitor (*rin*) mutant known to inhibit fruit ripening due to the natural deletion of *RIN* gene. However in this mutants, regulatory regions of *Macrocalyx* (MC) gene were lost, leading to distended sepals and loss of inflorescence determinacy (Vrebalov et al. 2002). Though inducing *rin* mutations through CRIPSR without affecting *MC* gene did not show multiple phenotypic affect (Ito et al. 2015), indicating precision in genetic editing.

In tomatoes Klap et al. (2017), knocked out Slagamous like 6 (*SlAGL6*) gene making mutants resulting facultative parthenocarpy under heat stress environments with regular shape and weight, maintained pollen viability, and retained sexual reproduction capacity. Simultaneously, Ueta et al. (2017) showed that by mutating *SlIAA9* gene of auxin signalling pathway, leads to seedless fruit development without fertilization. This accurate and rapid technique of developing parthenocarpy provides prospective for year round cropping. The knowledge can also be applied in other horticultural crops like watermelon, gourd or any elite cultivar etc. where seedless or less seeded fruits are in demand.

CRISPR (NHEJ) mediated deletion of the pathogen gene makes the plant unaffected to infections. This has been accomplished in tomato by targeting three mildew resistance locus (*MLO*) genes,(*SlMlO1*) gene contributing to susceptibility to fungi *Oidium neolycopersici* instigating powdery mildew disease (Nekrasov et al. 2017). Here, by using two sgRNA, *MLO* gene was targeted producing resistant transgene tomato. Analogous approach has been previously engaged to develop mildew resistant cultivar in wheat (Wang et al. 2014).

Wide range RNA virus resistance were established by silencing of *eIF4E* gene in melon and tomato (Mazier et al. 2011; Rodríguez Hernández et al. 2012). Likewise, virus resistant homozygous mutant cucumber was generated by deletion of *elF4E* gene with the help of two gRNAs targeted at two different sites (Chandrasekaran et al. 2016). The approach requires in depth genome data about susceptibility genes and produces transgene free mutants in contrast to reciprocal approach targeting viral DNA in plants resulting in virus resistant plants (Khatodia et al. 2017; Langner et al. 2018).

Starch purity, viscosity and binding strength in potato is an important factor for food as well as many areas like paper and adhesive industry. The “waxy genotype” (identified in maize) was created in hexaploid potato by repressing granule bound starch synthase (*GBSS*) gene disrupting amylose production limited to single wild type allele using CRSIPR (Andersson et al. 2017). DuPont Pioneer targets to commercialize a cultivar of corn with high amylopectin, higher yield that was established disrupting amylose biosynthesis gene *Wx1*using CRISPR (Waltz 2016). Similar multiallelic mutagenesis was performed in Acetolactate synthase 1 (*StALS1*) gene (Butler et al. 2016) & *StMYB44* which negatively regulate phosphate transport gene function making it perfect instrument for mutagenesis genetics (Zhou et al.

2017). Similarly in tomato significantly enhanced *GABA* (γaminobutyric acid) accumulation in both the leaves and fruits was achieved targeting five key genes which improved plant vegetative and reproductive growth (Li et al. 2018).

Damaged or cut white button mushrooms, turn brown on preserving. The reason behind mushroom browning is the presence of melanin pigment. *Yinong Yang*, from Pennsylvania State University a plant pathologist outlined the melanin production in mushroom to a specific polyphenol oxidase gene (*PPO*) and used CRISPR/Cas9 to delete6 of the *PPO* genes resulting non browning mushrooms. PPO has been targeted by companies developing non browning apples and potatoes (Waltz 2015a, b).

Stress tolerance and plant growth, are important agronomic traits regulated by hormones in plants. Jinrui Shi et al. 2017 at DuPont Pioneer used *ARGOS8*gene from a native corn variety and inserted it using CRISPR into non transgenic plants. *ARGOS8*mutant plants reduces sensitivity to ethylene, produced more bushels per hectare during drought conditions and maintained yield even under watery environments (Shi et al. 2017). Abscisic acid (ABA) is an important plant hormone regulating plant development. 9cisepoxycarotenoiddioxygenase (*NCED*) is the main rate limiting enzyme in ABA pathway with vital role in abiotic stress tolerance in rice. Overexpression of *OsNCED3* in transgenic plants could promote leaf senescence enhance water stress tolerance and increase ABA content, providing a new approach for improving the quality of crop (Hwang et al. 2018).

Miao et al. 2018, applied CRISPR/Cas9 to improve rice productivity by editing group I and group II *PYL* genes in rice. Studies on combinatorial mutants advised that group I genes have vital roles in guard cells stomatal opening and closing, delayed germination, and growth regulation when compared with group II. Remarkably, high order group I mutants, revealed the paramount development and enriched grain output in paddy fields environment, while keeping nearly normal seed dormancy.

CRISPR/Cas9 was also utilised in creating high oleic and low linoleic fatty acid content (FAD2) (Jiang et al. 2017; Abe et al. 2018); and also conferring resistance to Rice Tungro Disease (Macovei et al. 2018). Genes required for growth and development are also been targeted by CRISPR/Cas9 in other vegetables like *Brassica oleracea* (Lawrenson et al. 2015) and *Lactucasativa* (Woo et al. 2015). Introducing point mutations at specific genome position leads to enhanced agronomic characteristics conferring resistance to ALS and PSII inhibiting herbicides (Sun et al. 2016). ALS is a needed in biosynthesis of branched chain amino acids and essential herbicides including chlorsulfuron and bispyribac sodium (BS).

The start-up Arvegenix is using CRISPR tools to improve oil and meal quality in pennycress, a flowering plant from cabbage family. The goal is to make pennycress both a cover crop that is used between typical growing season's crops and a product similar to canola for the oil and feed markets. On similar lines crop genetics companies like Corteva (with Broad Institute), Pairwise Plants (with Monsanto), Benson hill biosystems, Calyxt, Cibus, Yield10 Biotech etc. are developing broad acre crops with benefits by this technology.

19.5.2 CRISPR in Fruit Crops

Genetic editing by CRISPR/Cas9 is developing more nutritious, colourful, tasteful and appealing fruits. Due to sluggish conventional breeding programs and due to its perennial flora comparative less attention was given to fruit improvement despite its high nutritional and economical value. Breeding periods of fruits have been lessened with the progress of marker assisted breeding, but it is still not adequate. Nevertheless, genome editing tools makes it now possible to edit the plant genome with precision.

There are, ample reports defining application of CRISPR/Cas9 in woody plants, such as oranges (Jia and Wang 2014a, b), apples (Malnoy et al. 2016) and grapes (Malnoy et al. 2016; Wang et al. 2016a, b).

The application of CRISPR where initially described in citrus crops by disrupting Phytoene desaturase (*CsPDS*) gene in carotenoid biosynthesis pathway for carotenoid accumulation (Jia and Wang 2014a, b). Jia et al. 2017 further used CRISPR/Cas9 for generating citrus canker resistant plants by disrupting gene *CsLOB1*(Lateral organ boundaries 1). Diverse alleles of *CsLOB1* gene in its promoter contain the effector binding element (*EBEPthA4*), which is recognized by *PthA4* of pathogen *Xanthomonascitri subsp. citri (Xcc)* to stimulate*CsLOB1* expression. Targeting *EBEPthA4*the effector binding element is an alternative to develop resistance to citrus canker (Peng et al. 2017). Additionally, genetic editing by CRISPR/Cas9 has been recognised in watermelon (Tian et al. 2017) and grapes (Nakajima et al. 2017) by targeting *PDS* gene in both crops, leading to visible bleaching phenotype changing the leaf colour from green to white.

CRISPR was applied to gene functional analysis in grapes by knockout of *VvWRKY52*transcription factor thereby increasing resistance to pathogen *Botrytis cinerea* (Wang et al. 2018). Interestingly, a grape specific database "Grape CRISPR" is already available (Wang et al. 2016a, b). These databanks provide brilliant support and decrease contextual work for recognising target locus in a genome.

Recently an alliance of Monsanto and Pairwise expects to introduce disease resistant sweeter strawberries with a longer shelf life. CRISPR can be used to edit the wild strawberry genome to improve traits with high efficiency (Zhou et al. 2018; Getaz et al. 2018). Okanagan Specialty Fruits publicised that it has positively completed the first marketable yield of its non browning Arctic Golden apple variety. The nutritious non browning fruit from this harvest will be sold as fresh cut apples in test markets across North America.

19.5.3 CRISPR/Cas9 in Other Horticultural Crops

Genetic editing by CRISPR/Cas9 is not confined exclusively to fruits and vegetables but also validated in other horticultural crops like flowers, spices, tuber crops, aromatic and medicinal plants. Not constrained to use of CRISPR/Cas9 with the woody plants, poplar (Zhou et al. 2015), the technology know how is also tested in non woody plants, like sorghum (Jiang et al. 2013) soya bean (Cai

et al. 2015) potato (Andersson et al. 2017) cotton (Li et al. 2017) and mosses (Collonnier et al. 2017).

Lotus Japonicus, a classical legume was first decorative plant tried for genetic editingthrough potential of CRISPR/Cas9. Here functional analysis of symbiotic nitrogen fixation genes was studied by creating multiple gene knockouts in nodules inactivating symbiosis receptor like kinase (*SYMRK*) and leghemoglobin loci (*LjLb1, LjLb2,* and *LjLb3*) (Wang et al. 2016a, b) using nodule specific LjLb2 promoter. Further illustration is of a economically valuable hexaploid decorative plant *Chrysanthemum morifolium*steadily expressing yellowish green fluorescent protein (*CpYGFP*) gene was genome edited by two different positioned sgRNAs (KishiKaboshi et al. 2017) enabling to monitor the progress of gene editing by help of fluorescence as well to understand mutation frequency around to the actual condition where multiallelic genes happen in the genome. Used in this study was *Petroselinum crispum ubiquitin* (PcUbi) promoter for endonuclease Cas9 and AtU6 promoter for sgRNAfor genetic editing. Chrysanthemum is natural source of insecticides and genetic editing of biosynthetic pathways, will lead to identification of efficient and comprehensive varieties of insecticides. Diterpene synthase gene (*SmCPS1*) in *Salvia miltiorrhiza*a chinese medicinal plant was disrupted using CRISPR generating deficient tanshinones mutants barring phenolics metabolites (Li et al. 2017). *Salvia miltiorrhiza*, has vital medicinal properties, such as vasorelaxation, protection against ischemia reperfusion injury, and antiarrhythmic effects. This editing technique will in addition open avenues for pathway elucidation of secondary metabolites, value improvement, and yield increases.

Genetic editing in medicinal flora will improve understanding the biosynthetic pathways of secondary metabolites. Thus, CRISPR/Cas9 can be utilized to metabolic engineering to enhance vegetables, fruits, and medicinal plants which are essential source of nutrition, health and wellbeing ancillary factors.

19.6 Safety Guidelines and Regulatory Specifications

Gene editing technology, CRISPR/Cas9, holds abundant potential for the development of science and many useful applications in plant biotechnology. More so with the increase of world's population and average temperatures, there is higher demand for more nutritious harvests and climate adaptable crops. Few genetically engineered products sailed through the US regulatory process, such as drought resistant corn, nonbrowning Arctic Apple, Simplot's Innate potato (Acrylamide free potatoes) etc. (Halterman et al. 2016). Although scientists are moving forward with CRISPR, others are apprehensive about the possibility that the use of CRISPR Cas9 can cause significant unintended gene mutations. CRISPR–Cas9 technology bypass product based GMO regulation because it generates transgene free plants through genetic editing. Scientific and legal experts have promoted that such non transgene crops should be non regulated, even under process centred GMO regulations (Hartung and Schiemann 2014; Camacho et al. 2014; Harvey 2014; European Academies' Science Advisory Council 2015; European Plant Science Organisation 2015; European Seed

Association 2015). Others disagree (GM Freeze 2016; GMWATCH 2014; Green Peace 2015; IFOAM EU 2015). Furthermore, researchers are apprehensive about CRISPR potential to bring changes to the guidelines of inheritance.

Governments around the world are just beginning to address this conundrum on NPBT also involving CRISPR. In March, 2018 USDA, together with the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA) judged that CRISPR modified crops (Non browning Arctic apples, Mushrooms, drought resistant corn)—and reviewed 30 other plants—do not succeed to regulate. USDA regulate GM crops primarily under 7 CFR Part 340 and Part 360, which imply that the risks associated with GM crops must be assessed and regulated based on the product and not on processes used in making the product (Araki et al. 2014; Araki and Ishii 2015). In Canada, GMO regulation is in belief not existing, and a different regulation vis-à-vis plants, with unique traits is existing, irrespective which technology was used to generate it (e.g., conventional breeding or mutagenesis, biotechnology) (The Plant Protection Act 1990).

Cisgenic plants are omitted from legislation of GMO's in Australia, while in Argentina, where the world's first regulation for NPBT was delivered in 2015, products without transgenes are to be assessed on individual basis (Ishii and Araki 2016). Brazil, India and China were considered to have process based regulations focused on the process of genetic modification (such as 'the genetic material has been changed by method that is unlike natural by mating and/or natural recombination') (Ramessar et al. 2008).

In Sweden, Swedish Board of Agriculture reasoned that arabidopsis varieties in which photosystem II (*PsbS)* was disrupted and a point mutation was introduced through CRISPR are not subject to regulations centred on the European Union (EU) definition of GMOs. In Germany, the government's risk assessment agency verified that a gene edited form of canola was not genetically modified and subsequently not subject to regulation supporting the application of NPBTs for future crop improvements. It ought to be noticed that this inference does not resonance conclusions from the EU. In EU there is indecision and the discussions on the legitimate elucidation of genetic editing techniques is very active. Chemical mutagenesis and ionizing radiation, causing random mutations in genome's, are at present excluded under the EU's GMO regulation. Perhaps, gene editing techniques that generate analogous products (but with greater precision) ought to likewise be absolved. In EU, GM crops are subject to regulatory evaluation through exhaustive methods under EU Directive 2001/2018/EC (2001) and EC Regulation 258/97 (1997), these are considered to be process based GMO regulations (Araki and Ishii 2015; Davidson 2010; Araki et al. 2014; Sprink et al. 2016). Presently, maize is the only GM harvest to be cultivated for commercial purposes in the EU. The European Food Safety Authority (EFSA) delivered two technical perspectives on latest breeding methods: (a) on the safety appraisal of plants generated through *cisgenesis* and intragenesis (European Food Safety Authority. 1. EFSA J 2012; 10: 2561) and (b) and safety evaluation of nuclease based genetic editing (EFSA J 2012; 10: 2943). EFSA presumed that the present rules on risk assessment of GM plants were suitable for cisgenic and intragenic plants, and furthermore for the nuclease based genetic editing

technique. EFSA is also careful to comprehend the hazards related with cisgenic plants similar to those identified as conventionally produced, but that unusual hazards could be concomitant with intragenic and transgenic plants. In January, 2018 EU directed several NPBTs like CRISPRCas to be likely exempted from investigation or scrutiny. Better clarity is expected by fall of 2018 as to whether crops produced through NPBTs must be regulated as GMOs. First, the Court of Justice of the European Union (CJEU) is expected to reach a decision on the 'mutagenesis exception' within Directive 2001/18/EC, which lawfully defines GMOs. Second, the European Commission is expected to issue its translation of whether NPBTs should fall inside the extent of GMO particular enactment (PurnHagen et al. 2018).

Genetic editing technology is emerging and enterprises are ardent on mechanisms to keep away from an inevitable bottleneck made by interlaced privileges and prolonged case. It will be of importance whether patents protected CRIPSR/Cas9 and other gene editing technology stays in effect and who will possess or acquire licenses to exclusionary rights. CRISPR/Cas9 has intricate, undecided patent landscape with numerous coinciding patents and patent applications on basic and advanced CRISPR/Cas9 approaches, new progresses or expansions. In addition to the two competing research groups (Feng Zhang & Jennifer Doudna), other research groups are also dominant. Recently (June, 2018), patents for the extensive use of CRISPR Cas9 for gene editing all types of cells have been issued to the DoudnaCharpentier team by the European Patent Office. The patent issued largely includes the use of a CRISPR Cas9 inside any type of plant or animal cell, or outside a cell, in order to modify a gene or the expression of a gene. Given the number of research teams working in the area, the intricacy of the patent landscape will possibly increase with time and generate further patent disputes.

Comprehensive civic society also creates progressively solid statements to governance of developing innovations, and there voice must be heard to indicate similitudes or differences amongst scientists and civic society with respect to healthiness, costs, benefits and safety issues of GM foods. In this way, it is mandatory on researchers involved in plant genetic editing to perceive that both regulators and civic society may enquire regarding genetic editing that might be considered impertinent to specialists in the field. Legislation needs to contemplate both the advantages and the challenging aspects of gene editing, from a larger public and value creating viewpoint. Researchers encounter these enquiries in phrases of regulatory processes for new products, governance of their research activities, and in participatory methods that organize intrigued parties. While the questions raised by gene editing are worldwide, legal guidelines and rules are to an inordinate extent inevitable by national borders.

19.7 Conclusions

Genetic editing is ready to change agriculture in the twenty-first century and beyond. The CRISPR– Cas9 has been utilized to enhance diverse crop attributes like high contents of plant secondary metabolites, increased grain yield,

importance of plant architecture, higher Shelf life of flowers, vegetables and fruits, stress tolerance, pesticide and herbicide resistance. We are now observing the underlying development of genome edited products, ranging from drought tolerant corn to non browning apple and mushrooms to soybean with improved oil attributes with assessments conducted by national authorities. With the continued use of CRISPR Casin crops, we will comprehend the imperatives and constraints of innovation and its futuristic effects.

CRISP technologies build in view of genetic editing permits concurrent changes in multiple genetic loci, which aid fast stacking of different characteristics in an elite variety background leading to noble crop varieties. The application of CRIPSR in remodelling Cas9 and Cpf1 for transcriptional regulation envisaging gene loci, distinguishing epigenetic modification and components driving promoter activity, and in addition in setting up contingent liaison between SNPs (single nucleotide polymorphism) determined by genome wide associate studies and eugenic traits. Nonetheless, data on plant genome sequences and gene functions is a prerequisite for conclusive genetic editing. Data on whole genome sequences will provide a significant jump towards key traits knowledge, enabling genome targeted breeding programmes and application of the latest advances in crop improvement, gene editing approaches. Critical tools for the disclosure of gene-trait affiliations are GWAS and QTL examination, which use genome-wide variation information and the phenotypes of crop populaces. Expanding the focus of trait studies from model harvests, for example, rice to non-model crops and crop relatives opens up an essential assorted breeding.

Vital logical requirements around there is to have enhanced strategies to foresee and affirm off target effects within the genome to unexpected downstream effects of genome edited crops or to ecosystems or to consumers of inferred products. There is a need to enhance the predictability of off target modifications to gain more extensive acknowledgement. Tending to this will require further advances in genomic and other omics techniques and their translation. Improvements in bioinformatics and the expanding accessibility of complete genome maps are helping WGS to achieve robustness at costs and analytical timespans that allow for routine utilization of WGS as a portrayal device. In principle, these strategies ought to distinguish mutations happening throughout an edited genome, yet at present have limited reliability because of incomplete sequence coverage and alignments, factual vulnerabilities in genome assembly techniques for families of small size reads, and the challenges of dealing with repetitive regions.

In the long run, the regulatory course forward for gene edited plants is presently caught in semantic and statutory contentions in regards to display regulatory methods for GMOs. Those assessments ought to be tended if there's to be a longhaul respond in due order regarding plant bioengineering, on the grounds that the nature and way of development will potentially outpace regulatory concepts that are founded on process contemplations. Understanding risks and vulnerabilities related to procedures for genome engineering is essential, once understanding builds in science community on technological know-how and is also subject to social talk, a definitive administrative benchmarks for engineered crops need to centre around the

properties of the harvest phenotype. Albeit numerous challenges are there to determine, however these obstacles will be expelled and the efficient, highly precise genetic editing CRISPR/Cas innovation, will absolutely be consolidated in plant breeding.

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Defense Mechanism and Diverse Actions of Fungal Biological Control Agents Against Plant Biotic Stresses

20

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Abstract

The terms "biological control" or its abbreviation "biocontrol" is an environmentally sound and effective means of mitigating or reducing of pathogen/pests by interference with their ecological status, as by introducing a natural enemy into their environment. The concept of biological control have been used in various fields of research, most notably plant pathology and entomology. In plant pathology, it has been used to describe the use of microbial antagonists to suppress plant diseases. In entomology, the term applies to the use of insects' predators, or microbial agents to suppress populations of various pest insects. In both fields, the living organism that used to suppresses the pathogen or pest is referred to as the biological control agent (BCA). With regards to plant pathology, the plant host responds to various environmental stimuli, including non-pathogenic and pathogenic, thus induced host resistance, considered outstanding formula of biological control. In this chapter we will discuss the mechanism and mode of actions of different fungal BCA for induction of crop disease resistance and specific advantages of using this control method in integrated disease management (IDM) in crop plants.

Keywords

Biocontrol agents · Trichoderma spp · Induced systemic resistance · Different mechanism

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20.1 Introduction

Agricultural crops are frequently affected by various biotic and abiotic stresses and the magnitude of the stress effects depend on the nature of the causes and the duration of expose. In addition, world food production will have to rise by 70% to meet the expected increase of world population to 9 billion by 2050 (Stratonovitch and Semenov 2015). The increasing pressure of population growth combined with severe crop losses due to environmental stresses imposes the urgent need for sustainable strategies to improve food security (Bita and Gerats 2013). Biological control is an ecofriendly profitable and a reliable method for crop protection. Therefore, it plays a crucial role in the development and assessment of plant protection methods, in situations where chemical/physical control were either insufficient or undesired due to health and environmental concern. Despites chemical control is rapid and effective method, it has a high toxic component that cause a serious health and environmental problem including carcinogenic, high residual effect and longtime of degradation (Tripathi and Dubey 2004; Guédez et al. 2010). Therefore, the current trend is to reduce the use of chemical control and switch to biological control as potential method for plant disease protection in vast number of cropping systems. The main reason for biological control increasing popularity is the safety record and the longer stability in the ecosystem compared with physical and chemical approaches (Sharma et al. 2013). The new tools of recombinant DNA technology by using gene modification of the BCA combined with a traditional approaches such as breeding program and improved germplasm should speedily move the biological control research and technology into a new epoch.

Soil biodiversity plays an essential role in the sustainability of agriculture ecosystem especially when considering the soil richness with different microbial life forms that are involved in biological control of soil borne diseases (Gil et al. 2009). The interaction between these beneficial microorganism and plants, facilitates plant growth and enhance plant defense against various pathogens (Jogaiah et al. 2013; Anupama et al. 2015; Abdelrahman et al. 2016; Jogaiah et al. 2018). Several conceivable mechanisms have been proposed to explain this microbial beneficial response on plant growth, including control of deleterious root microorganisms, direct production of plant hormones, and increase of nutrient uptakes specially phosphorus and nitrogen (Ishihara et al. 2008; Jogaiah et al. 2013; Abdelrahman et al. 2016; Jogaiah et al. 2018). Plant growth promoting rhizobacteria (PGPRs) and fungi (PGPFs) have been considered more natural and environmentally acceptable BCA for managing the disease and growth parameters in a number of vegetable crops (Jogaiah et al. 2013; Anupama et al. 2015; Abdelrahman et al. 2016). Among PGPFs, the species of *Trichoderma* are most commonly used in agriculture system (Van Wees et al. 2008; Shoresh et al. 2010; Jogaiah et al. 2018). Root colonization by *Trichoderma* spp. causes substantial changes to the plant proteome and metabolome, resulting in tolerability to pathogen and improvements of the crop productivity (Harman et al. 2004; Shoresh et al. 2010). Several *Trichoderma* species such as *T. harzianum*, *T. lignorum*, *T. atroviride*, T. *longibrachiatum* and *T. virens* were applied as BCA against fungal pathogens and nematodes (Spiegel and Chet 1998;

Lahlali and Hijri 2010; Abdelrahman et al. 2016). The considerable variability among different fungal BCA has drag the attention into the importance of the screening and selection criteria of the BCA.

20.2 Isolation and Identification of Fungal Biological Control Agent

Fungal BCA are non-pathogenic organisms that exhibit antagonistic nature against microbial pathogens including bacteria, fungi, and nematodes that cause various economically serious diseases in many cropping systems (Sahebani and Hadavi 2008; Perazzolli et al. 2011; Brotman et al. 2013; Jogaiah et al. 2013). The effectiveness of fungal BCA in controling the incidence and strength of disease severity caused by microbial plant pathogens has been reported in numerous studies (Shoresh et al. 2010; Jogaiah et al. 2013; Abdelrahman et al. 2016; Jogaiah et al. 2018). Fungal BCA exhibit a considerable variation in their biocontrol efficacy depending on the applied strains or subspecies. Theses variability is mainly due to the differences in their rhizosphere competency, sensitivity to environmental conditions, nature and quantity of bioactive metabolites and enzymes produced by these BCA. Therefore, it's important to precisely isolate and identify a potential BCA though various biochemical and molecular characterization. After identification of the isolated fungal BCA, their biocontrol capacity has to be evaluated by various screening techniques using *in vitro* and greenhouse conditions to choose the most active species or strain for further applications under field conditions.

20.2.1 Culture Methods

Fungal BCA can be isolated using suitable medium that favor its growth and provide the essential nutrients for their development. Several fungal BCA can be grown on general media such as potato dextrose agar (PDA), but some may need selective media for their growth. The optimum growth conditions including pH, temperature, carbon and nitrogen (C:N) ratio and light intensity are essential factors for the fungal BAC mycelial growth, sporulation, production of enzymes, antibiotics and other bioactive metabolites that involved in their biocontrol activity against pathogens (Waghunde et al. 2016). pH is a key factor to manipulate growth and sporulation of *T. harizanum*, while C:N ratio strongly affected spore formation time (Agosin et al. 1997). The isolation and quantification of microbial BCA by plate count, is one of the most commonly used techniques to assess soil biodiversity (Hirte 1969). Plate count methods has some benefits over non-culture techniques, as it facilitates genetics and taxonomic studies of the isolated microorganisms (Vieira and Nahas 2005). Several soil *Trichoderma*-selective medium (TSM) have been described (Table 20.1). For instance, Elad et al. (1981) developed TSM consisted of the following ingredients (g per liter distilled water): 0.9 g of $K_2 HPO_4$; 0.2 g of $MgSO_4.7H_2O$; 0.15 g of KC1; 1.0 g of $NH_4 NO_3$; 3.0 g of glucose; 0.25 g of chloramphenicol; 0.3 g of

Table 20.1 *Trichoderma* selective media and substrates for growth and mass production

Trichoderma species	Selective media	Reference
T. harizanum	*Trichoderma*-selective medium (TSM)	Elad et al. (1981)
T. harzianum	TSM modified with antifungal and antibacterial components	Williams et al. (2003)
Trichoderma spp.	TSM modified with baytan, benomyl and Triton X-100	Hetong et al. (2005)
Trichoderma spp.	Potato dextrose agar (PDA) medium modified with rose Bengal, chloramphenicol and streptomycin sulfate	Gil et al. (2009)

p-dimethylaminobenzenediazo sodium sulfonate; 0.2 g of pentachloronitrobenzene; 0.15 g of rose-Bengal; 20 g of agar, as an effective selective medium for *Trichoderma* isolation. Further, Williams et al. (2003) developed TSM selective media consisted of 0.9 g of K_2HPO_4, 0.2 g of $MgSO_4.7H_2O$, 0.15 g of KCl, 1.0 g of NH_4NO_3, 3 g of glucose, 0.15 g of rose Bengal, and 20 g of agar in 950 ml of distilled water, which was autoclaved at 121 °C for 15 min. The antimicrobial and fungicidal ingredients (all amounts are per liter) were 9.0 ml of streptomycin stock solution (1% w/v), 0.2 g of quintozene, 0.25 g of chloramphenicol, and 1.2 ml of propamocarb (772 g of active ingredient per liter), all in 40 ml of sterile distilled water, and the mixture was added to the cooled basal medium. In addition, PDA medium supplemented with benomyl to stimulate the vegetative growth of *Trichoderma*, Baytan to promote *Trichoderma* conidia formation and Triton X-100 to limit the colony size has been reported as TSM suitable for isolation and quantification of *Trichoderma* spp. (Hetong et al. 2005). These selective media can eliminate the growth of other soil microorganisms and enable the growth of selective fungal BCA. Further cultivation methods of fungal BCA was continuously developed to achieve maximum growth. After achieving the optimal growth, the developed fungi are examined under microscope to examine their morphological characteristics including sporangia, conidia and spore structures, which need a considerable knowledge in mycological taxonomy.

One of the greatest obstructions to *Trichoderma* BCA is the limitation of mass culturing techniques. Recently, agricultural wastes like sugarcane bagasse powder were used as a growth medium for *T. harizanum*, which enhanced the growth and sporulation rate and suppressed *Rhizoctonia solani* causing damping-off disease in chilli (*Capsicum annuum* L.) seedling (Subash et al. 2014). Additionally, several *Trichoderma* spp. have been grown on various solid based substrate and grains such as sorghum (*Sorghum bicolor*) grains, barely (*Hordeum vulgare*) grains, wheat (*Triticum* spp.) straw, wheat bran, coffee (*Coffea* spp.) husk, wheat bran-saw dust, for their mass production and acceleration of sporulation (Waghunde et al. 2016) (Table 20.2). The utilization of agriculture wastes as substrate for the mass production of *Trichoderma* BCA had several advantages including low energy cost, less expensive processing, high reproducibility and easier control of contamination (Waghunde et al. 2016).

Table 20.2 *Trichoderma* spp. grown on various agriculture wastes and solid based substrate and grains

Trichoderma species	Agriculture wastes and solid based grain	Reference
T. harzianum (T5), T. viride, T. hamatum (T16)	Cotton cake	Sharma and Trivedi (2005)
T. harzianum and T. viride	Cow dung with neem cake and coir pith	Rini and Sulochana (2007)
T. harzianum	Rice bran, paddy straw, groundnut shells	Parab et al. (2008)
T. harzianum	Maize grain	Pramod and Palakshappa (2009)
T. harzianum	Sorghum grain	Upadhyay and Mukhopadhyay (2009)
T. viride	Wheat and sorghum grains	Bhagat et al. (2010)
Trichoderma harizanum	Sugarcane bagasse	Subash et al. (2014)
T. harizianum	Jatropha cake and neem cake	Tomer et al. (2015)

20.2.2 Molecular Characterization of Fungal BCA

Identification of fungal BCA based on morphological characteristics is not enough because of the high similarity of the morphological features among species. Therefore, additional tests are need to differentiate between various fungal BCA. Molecular techniques of fungal BCA identification and differentiation provide a high level of accuracy and specificity. The molecular identification based on the internal transcribed spacers (ITS) of ribosomal DNA is one of the most effective and reliable method for identification of fungal BCA (Jogaiah et al. 2013; Abdelrahman et al. 2016). For example, *Pythium contiguanum* isolated from the salt-march in Algeria exhibited high morphological characters with phytopathogens *P. periilium.* However, the ITS sequence revealed a differential homogenous in their nucleotide sequence between *P. contiguanum* and other *Pythium* species including *P. periilium* (92%), *P. torulosum* (86%) and *P. vanterpoolii* (78.5%) which enable to classify *P. contiguanum* into a new taxon. Additionally, *P. contiguanum* was found to exhibit biocontrol activity against *Botrytis cinerea* causing gray mold disease in grapevine (Paul 2003). Two *Trichoderma* T1 and T2 isolates obtained from the desert soil of Egypt was identified using ITS region revealing high similarity with *T. longibrachiatum* (Abdelrahman et al. 2016). Further evaluation of the isolated *T. longibrachiatum* BCA showed antagonistic nature against *Fusarium oxysporum* pathogen in bulb onion (*Allium cepa*). Characterization of 16 biocontrol strains was carried out using several molecular techniques (Hermosa et al. 2000). Further, phylogenetic study based on ITS1 sequences, clustered the 16 BCA into four groups: *T. longibrachiatum*, *T. harzianum-T. inhamatum* complex, *T. asperellum* and *T. atroviride-T. koningii* complex. Interestingly, none of the identified BCA corresponded to Th2 or Th4 of *T. harzianum*, which cause mushroom green mold (Hermosa et al. 2000). Isolation and identification of important genes related to biocontrol activity could be an important feature for marker development-based selection of different

BCA. Identification of an ATP-binding cassette transporter cell membrane pump as an important component manipulate the antagonism and biocontrol mechanisms of *Trichoderma* spp. (Ruocco et al. 2009). The encoding gene, named *Taabc2*, was found to be upregulated in the presence of pathogen-secreted metabolites, specific mycotoxins and in conditions that stimulate the production in *Trichoderma* spp. of antagonism-related factors (toxins and enzymes). Moreover, two new ketoacyl synthase (KS) fragments were isolated from cultured *T. harzianum* mycelia and these genes are differentially regulated in *T. harzianum* challenged with pathogens (Yao et al. 2015). Further analysis revealed that these genes are important for RAL biosynthesis and responsible for conidial pigmentation during antagonistic which can be used for BCA differentiation (Yao et al. 2015).

20.2.3 Biochemical Methods

Biochemical assay are now frequently used along with morphological and molecular characteristics to differentiate the fungal BCA based on their biocontrol capabilities. Dual culture assay is an effective techniques to measure *in vitro*, the antagonistic nature of fungal BAC against phytopathogens (Abdelrahman et al. 2016). In this technique agar disc (5 mm) was taken from 5-day-old PDA culture plates of the selected fungal BCA isolate and placed at the adjusted margin of the PDA plates. Another agar disc of the same size of fungal pathogen is placed on the same distance but on the opposite end of the same Petri dish (Fig. 20.1a). Antagonistic activity is calculated based on the percentage inhibition of radial growth relative to control (Fig. 20.1b).

Another method for measuring the antagonist activity of the fungal BCA is to use the fungal BCA culture filtrates (Rahman et al. 2009; Siameto et al. 2010). These BCA culture filtrates were collected after considerable period of incubation, filtered using sterilized membrane filter and concentrated using a vacuum evaporator. PDA medium supplemented with different concentration of concentrated culture filtrates will be used for screening the antagonistic activity against different phytopathogens. Radial extension of the mycelia on culture plates for both the experimental

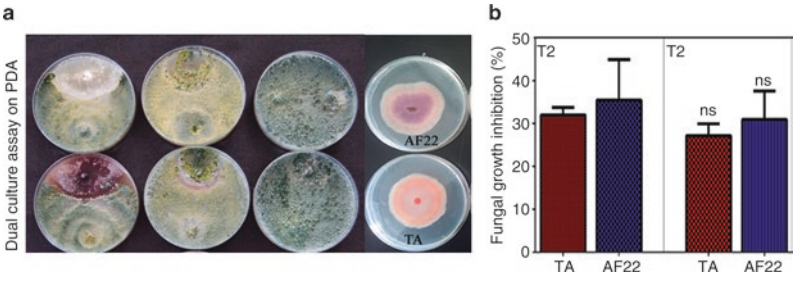

Fig. 20.1 Dual culture antagonistic assay. (**a**) Potato dextrose agar (PDA) plate's antagonism of *Trichoderma* strain T1 and T2 against different strains of *Fusarium oxysporum* f. sp. *cepa*. AF22 and TA. (**b**) Fungal growth inhibition percentage

treatment and control will be recorded and inhibition of radial growth will be calculated. In addition, antagonistic activity of the fungal BCA can be evaluated by microplate assay of the enzymatic activities of β-1,3-glucanase an important cell wall degrading enzymes for many *Trichoderma* spp. (Ramada et al. 2010). Root colonization ability is an essential factor for determination of fungal BCA efficacy (Salas-Marina et al. 2011). Roots of the host plant-inoculated with fungal BCA are surface sterilized with sodium hypochlorite for 3 min and placed on a Petri dish with MS or PDA medium. Plates were visually inspected to evaluate the fungal BCA growing mycelium evolving from the root. Further inspection can be also carried out using root fragment post-inoculated with fungal BCA, stained with WGA-AF 488 (Molecular Probes) according to Deshmukh et al. (2006). Confocal laser scanning microscope used for detecting the fluorescence signal from stained hyphae in the root fragments (Andrade-Linares et al. 2011).

Phosphorus solubilization is one of the most important feature of *Trichoderma* BCA and common technique used for fungal BCA differentiation (Jogaiah et al. 2013, Abdelrahman et al. 2016). Fungal BCA cultures were grown in Pikovskay (PVK) broth for considerable incubation time and soluble phosphorus content was determined according to Watanabe and Olsen (1965). Phosphorus solublization by fungal BCA will facilitate nutrients uptake which improve plant growth under control and infected conditions (Jogaiah et al. 2013, Abdelrahman et al. 2016). Additionally, induction of indole acetic acid (IAA) by fungal BCA as key hormones that enable plant growth and trigger plant defense is common technique for fungal BCA differentiation (Jogaiah et al. 2013; Fu et al. 2015; Abdelrahman et al. 2016). The amount of IAA produced by fungal BCA was determined using potato dextrose broth supplemented with 1 mg l-tryptophan ml^{-1} and incubated at 26 °C for 5 days. After incubation, the inoculated medium was centrifuged and the supernatant was used for the determination of IAA according to Gordon and Weber (1951).

20.3 *Trichoderma* Diversity as Biological Control Agent

Trichoderma species are generally considered for their potential as BCA against different plant pathogens and several *Trichoderma* BCAs have been extensively studied using different cropping systems (Jogaiah et al. 2013; Abdelrahman et al. 2016; Jogaiah et al. 2018). *Trichoderma harzianum* T-22 is registered as BCA of many diseases in different crops, and it can be applied for seed priming or directly on the soil (Harman et al. 2004). *Trichoderma* species have been identified from a diverse range of natural and artificial habitats including soil, woody materials and marine, which demonstrates their high opportunistic potential and adaptability to various ecological niches (Druzhinina et al. 2011). *Trichoderma* spp. are belonging to one of the most useful group of fungus that have a key impact on the human welfare. They are recognized as the most widely used plant growth modifiers, biocontrol, sources of enzymes of industrial application, producers of secondary metabolites, clinical significance and microbial cell factories for important protein production (Harman 2011; Jogaiah et al. 2013; Abdelrahman et al. 2016).

Trichoderma-plant interaction is not strict only on their symbiotic properties as with rhizobia and mycorrhiza, but yield improvement and antagonistic nature against soil borne pathogens are more striking (Mukherjee et al. 2013). The environmental factors, plant genotypes and *Trichoderma* species- or strain-specificity can greatly affect the biocontrol activity and abundance, therefore biodiversity and phylogeny of *Trichoderma* is needed (Battaglia et al. 2013).

Since the introduction of the molecular tools in the diversity and taxonomy of *Trichoderma*, the number of species has dramatically increased (Jaklitsch 2011). The molecular ecology and genomics studies indicate that the presence of *Trichoderma* in soil, as biotroph or saprotroph, could be driven by the general mycotrophy, including various forms of mycoparasitism, combined with broad environmental opportunism (Friedl and Druzhinina 2012). The taxonomy of *Trichoderma* species is now recognized as a combination of phenotypic, phylogenetic as well as biogeographical extent (Mukherjee et al. 2013). Diversity and phylogeny of the *Trichoderma* genus have experienced tremendous refinement at the species level, and this is because of the development of the phylogenetic molecular markers (Jaklitsch and Voglmayr 2013). In 2005, Druzhinina and Kubicek reviewed the phylogeny and the species number of *Hypocrea*/*Trichoderma* species with 88 taxa, out of which 14 were recognized as holomorphs, whereas 49 were described as *Hypocrea* and 25 were named as *Trichoderma*. Recently, the species molecular data raised the species number into 194 species, of which 84 are *Holomorphs*, 48 *Hypocrea* and 62 *Trichoderma* (Atanasova et al. 2013). Detailed analyses of the taxonomic purposes suggested that continues usage of the DNA-directed RNA polymerase II subunit (*rpb2*), translation elongation factor 1(*tef1*) large intron and last large exon, Internal transcribed spacer (ITS1 and ITS2) and the endochitinase (chi18-5) last large exon as diagnostic molecular markers may led to more reliable phylogeny (Druzhinina and Kubicek 2005; Atanasova et al. 2013).

Trichoderma is a hyper-diverse genus and most of the previous studies on the, diversity, taxonomy and phylogeny of *Trichoderma* were based on random collections (Chaverri and Samuels 2004; Overton et al. 2006). However, designed diversity studies concentrated on soil-inhabiting species in geographically limited areas are recently being invested (Jaklitsch and Voglmayr 2013). Mulaw et al. (2010) reported eight putatively new species from 134 isolates from the *Coffea* rhizosphere in Ethiopia. Six species among 201 isolates from rice filed in Iran were reported (Naeimi et al. 2011). Błaszczyk et al. (2011), extended the soil studies by including compost, wood, and cereal grains detected 14 species in 170 isolates in Poland. Sun et al. (2012) reported 23 species from massive number of 1910 soil samples collected in China. Al-Sadi et al. (2015) detected 52 species from 194 soil samples from Oman. Many of these studies relied on ITS marker and their soil studies indicated that *T. harzianum* is the predominant species cluster in that habitat. Jaklitsch and Voglmayr (2013) represented 636 identified specimens and isolates were all allocated in *Trichoderma* clad phylogeny and *T. viride* represented by 43–62% of all isolates, dominates in regions with Mediterranean climate. The research scheme focused on the ecology and biodiversity of *Trichoderma* structural population in soil

and rhizosphere environment is important factor for sustainable agriculture and improve the cropping system (Maina et al. 2015).

20.4 Evaluation of the Usefulness and Effectiveness of *Trichoderma* as Biological Control Agents

The ecological characteristics of *Trichoderma* spp. is well-suited for its applications as BCAs of various fungal diseases. *Trichoderma* species are widely distributed in different soil types, especially in the habitats with rich plant debris and decaying wood (Druzhinina et al. 2011). In addition, *Trichoderma* spp. have a great capability to colonize different environment because they can grow very rapidly with low nutritional requirements and can survive under thrilling conditions. *Trichoderma* spp. have a very diverse metabolic capability by converting a wide range of organic matters through extracellular enzymes that degrade different types of polymers, as well as solubilization of minerals such as phosphorus, iron and copper, into achievable nutrient form by plants (Altomare et al. 1999; Schmoll et al. 2010; Jogaiah et al. 2013). All these features endorse the effectiveness of *Trichoderma* as potential BCA. Examples of successful application of *Trichoderma* BCA in different cropping system are given below.

Application of various concentrations of the *T. asperellum* T34 as BCA against root rot disease caused by *Phytophthora capsici* in pepper (*Capsicum annuum*) has been documented (Segarra et al. 2013). *Trichoderma harzianum* and *T. viride* were effective BCAs in reducing the root-knot nematode disease incidence in tomato plant caused by *Meloidogyne incognita* (Dababat et al. 2006). Novel isolates of *T. asperellum* with efficient antagonistic capacity against *F. oxysporum* f. sp. *lycopersici* (FOL) showed a promising alternative strategy for tomato wilt disease management (El_Komy et al. 2015). *Trichoderma viride* showed mycoparasitism and destructive control against two fungal soil pathogens, *F. oxysporum* f. sp. *adzuki* and *Pythium arrhenomanes*, and simultaneously increased the growth of soybean (*Glycine max*) plant (John et al. 2010). Desert soil *T. longibrachiatum* T1 isolate exhibited a significant control over bulb onion basal rot disease caused by *F. oxysporum* f.sp. *cepa* under greenhouse conditions (Abdelrahman et al. 2016). Field trails are essential to assess the efficacy of the selected species/strain of BCA which exhibited effective biocontrol features in vitro or under greenhouse conditions.

The effective role of *Trichoderma* as BCA of various pathogens can be divided into indirect and direct effect (Munir et al. 2013; Al-Naemi et al. 2016). Indirect effect include the activation of the plant defense systems (Jogaiah et al. 2013; Abdelrahman et al. 2016). Direct effects include the inhibition of the pathogen enzymes, competition for nutrients and space, the secretion of bioactive metabolites to suppress the pathogen (Anita et al. 2012). In the following section we will discuss in detail the biological control mechanism used by *Trichoderma* spp.

20.5 Mechanisms of *Trichoderma* Biological Control-Induced Resistance in Host Plant

20.5.1 Triggering of the Defense Mechanisms of the Host Plant

The main focus of *Trichoderma* BCA study was to understand their effects on the host plant defense response against various pathogen systems. *Trichoderma harzianum* strain T-22 is the famous BCA stated to induce systemic resistance (ISR) against pathogens in model plants as well as crops which highlighted its unique capacity (Contreras-Cornejo et al. 2011; Harman et al. 2012; Yoshioka et al. 2012). Other *Trichoderma* spp. were also reported such as *T. atroviride* and *T. virens* induced ISR in tomato against foliar pathogens *Botrytis cinerea* and *Alternaria solani* (Salas-Marina et al. 2015). Therefore, ISR is suggested to be one of the most significant mechanisms that regulates the biocontrol effects of *Trichoderma,* which is closely associated with the accumulation of ethylene/jasmonic acid, and the transcription of ISR-related genes (Harman 2006). Activation of ISR against phytopathogens by *Trichoderma* has been reported for both monocot and dicot plants (Djonovic et al. 2007; Shoresh and Harman 2008). Accumulating evidences indicate that ISR induced by *Trichoderma* spp. occur at the early stage of root colonization, where *Trichoderma* is recognized as foreign organism, through its microbial-associated molecular patterns (MAMPs), leading to the induction of ISR (Hermosa et al. 2013). The signaling molecules that involved in the manipulation of the ISR suggested to be the hydrolytic enzymes, oligosaccharides, low molecular weight compounds secreted by *Trichoderma* (Dean et al. 1989; Salas-Marina et al. 2015). Additionally, Induction of systemic acquired resistance (SAR) which is phenotypically similar with ISR but significantly different at the genetic and biochemical levels has been reported in several plants primed with *Trichoderma* (Contreras-Cornejo et al. 2011; Salas-Marina et al. 2015). *Trichoderma atroviride* induced the expression of SAR-related genes including *pathogenesis-related proteins* (*PR-1a* and *PR-2*) in *Arabidopsis* seedling to confer resistance against foliar pathogens *B. cinerea* (Salas-Marina et al. 2011). ISR response involves the induction of defense-related enzymes such as peroxidases, proteases and chitinases followed by lignification of cell wall around the site of the infection to suppress the pathogen spread (Mejía et al. 2008). Several reports have been conducted to assess the level of the induced defense-related enzymes in response to *Trichoderma* BCA before and after pathogen inoculation. For example Jogaiah et al. (2013) reported a significant increase in phenylalanine ammonia lyase, peroxidase and β-1,3-glucanase in tomato seedling primed with *T. harzianum* TriH_JSB27 strain. In addition, expression analysis of tomato ISR defense-related genes including *endochitinase*, *peroxidase*, *α–dioxygenase* were up-regulated in tomato seedling primed with *T. virens* (Salas-Marina et al. 2015).

Several *Trichoderma* spp. including *T. longibrachiatum*, *T. asperellum*, *T. virens*, *T. atroviride* and *T. harzianum* induce metabolic changes that confer higher resistance against many plant-pathogens (Waghunde et al. 2016). *Trichoderma longibrachiatum* induced many primary and secondary metabolites including carbohydrates,

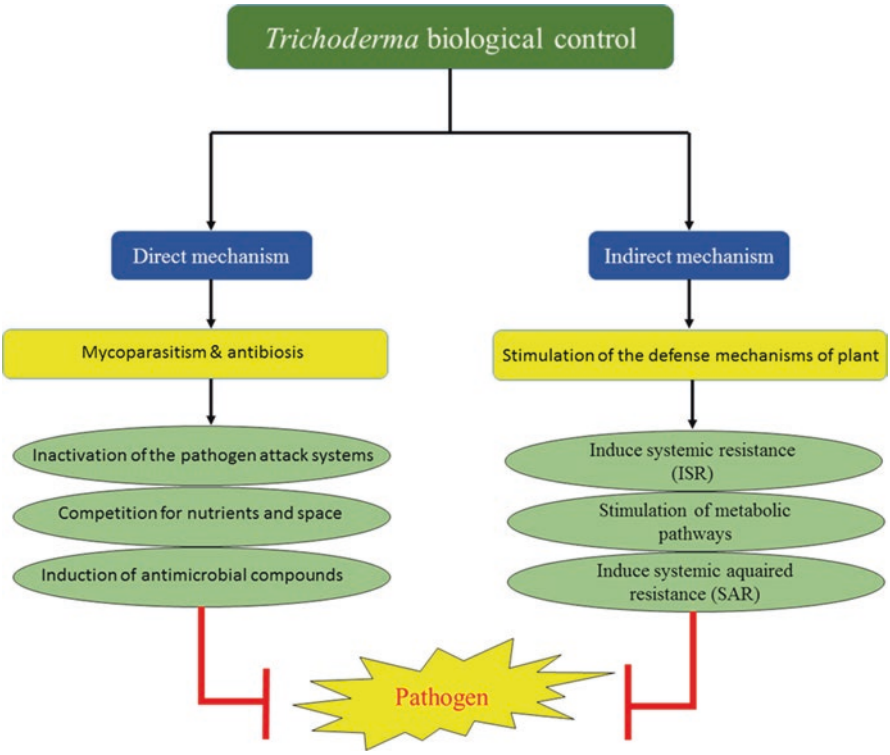

Fig. 20.2 Schematic diagram of the *Trichoderma* biological control mechanisms

phenylpropanoid and sulfur assimilation pathways in onion against *F. oxysporum* f.sp. *cepa* (Abdelrahman et al. 2016). In addition, *Arabidopsis* root colonization by *T. asperelloides* induced plant metabolic profile, including significant changes in polyamines, amino acids, sugars and citric acid (Brotman et al. 2012). This metabolite dynamics reflected as an increase in energy supply needed for the induction of plant defenses and growth promotion as a consequence of Trichoderma priming effects (Brotman et al. 2012) (Fig. 20.2).

20.6 The Use of Biological Control Agent for Pest Management

Decreasing the crop losses through ecofriendly pest management strategies will increase food availability and boost economic growth, especially in the developing countries where expensive approaches are not suitable (Grzywacz et al. 2014). The application of *Trichoderma* BCA to manipulate crop pest destructive effects have been reported recently. The application of neem leaves with *T. harizanum* BCA was effective approach to reduce the suppressive effect of root-knot nematode

Meloidogyne incognita in egg plants, leading to a significant increase in the yield production of the treated plants compared with untreated one (Khan et al. 2012). Moreover, the application of mixture of *T. harzianum* and *T. viride* significantly suppress the nematode (*M. incognita*) populations in the media and number of galls formation in the queen palms (*Livistona rotundifolia*), which indicates the efficacy of *Trichoderma* species to control root knot nematode (Jegathambigai et al. 2008).

The biocontrol of pests relies on, parasitism, predation, herbivory, or other natural mechanisms. Therefore, integrated pest management combines all the effective and environmentally sound methods into a single elastic strategy to manage pests. Insect predators and parasitoids such as parasitic wasps and entomophagous beetles, botanical pesticides that may poison the pest or make the crop unpalatable to them (pyrethrum and neem products) as well as plants that indirectly manipulate pest populations via natural products could be an effective way to control crop pest (Isman 2006; Khan et al. 2010; Grzywacz et al. 2014). The application of biocontrol in the filed for pest management relies on three major techniques: (1) conservation of present natural enemies, (2) hosting new natural enemies and (3) mass rearing and periodic release, based on a seasonal basis or inundative release (Van Driesche and Bellows 1996). An example of successful application of natural enemies is the use of the parasitoid wasp, *Encarsia formosa* Gahan to suppress the population of whitefly, *Trialeurodes vaporariorum* a ubiquitous pest of vegetable and floriculture crops which is difficult to manage even with pesticides (Hussey and Scopes 1985, Parrella 1990). Moreover, several types of insect predators such as ladybird beetles, carabid (ground) beetles, lacewings, minute pirate bugs, and spiders, can be found in varied habitats and they are mostly generalist predators, however some insect predators can be specific natural enemies by attacking one or few species of prey (Barbercheck 2011). Pesticidal and deterrent plant species provide prospective solutions against many major agricultural pests (Stevenson et al. 2012). For example *Bobgunnia madagascariensis* have been reportedly used for pest control, however, variation in the compounds associated with its biological activity at different geographical zones needs to be monitored to ensure their efficacy (Sarasan et al. 2011). Additionally, soil-surface dwelling agents using Miscanthus x giganteus (Mxg) shelterbelts as refugia provide potential BCA rich communities (*Phalangium opilio*, centipedes, predatory beetles and *Dicyrtoma fusca*) that can be implemented in insect pest management in agroecosystems (Morgan 2015).

20.7 Conclusion

Expected increase of world population imposes the urgent need for sustainable strategies to improve food availability. Therefore, biologically-based pathogen/pest management practices represent a potential and effective alternative for traditional agriculture practices. Suggesting that the market potential of BCA will increase in upcoming years. Different BCAs have been applied successfully under greenhouse and filed conditions. Cooperative and extension research programs on the mechanisms of BCAs using different species/strains within various cropping systems, will

ensure that future success and innovations in biological control research will continue and fulfill the market interests. The advanced DNA technology by using gene transformation of the BCA to increase favorable traits combined with breeding program through selective germplasm should rapidly move the biological control research and technology into a new era.

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Part IV

Disease and Crop Management

ROS Mediated Plant Defense Against Abiotic Stresses

21

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Abstract

Changes in the natural environment may cause stress to living organisms. This phenomenon has been widely observed in plants during abiotic stresses which include changes in temperature, pH of the soil, salinity and water content. Such stresses usually lead to the production of reactive oxygen species (ROS) in plant cells. Although ROS are generally produced inside plant cells as a by-product of different metabolic pathways including electron transport in the process of respiration, photosynthesis and several other chemical reactions, excessive production of these free radicals can lead to damage of the cell components due to oxidative stress. Other means of ROS production include external stimuli including biotic and abiotic stresses or cell lignification processes which lead to the enzymatic production of ROS. Various forms of ROS may include hydrogen peroxide (H_2O_2), hydroxyl radical (HO·), singular oxygen ($^1O_2$), and superoxide ion ($O_2\cdot^-$). These highly reactive free radicals are produced in different cellular compartments such as mitochondria, chloroplasts, peroxisome and the apoplast. Key roles for various reactive oxygen molecules include involvement in plant growth and development, programmed cell death, acclimation of stresses such as drought, salt, heat, cold, light and high frequency electromagnetic fields. In

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addition to involvement in stress acclimation and mitigation ROS serve as signaling molecules during biotic and abiotic stress responses. ROS are also involved in cross talk between different phytohormones, calcium signaling, antioxidant proteins, protein *kinases* and *phosphatases* and map *kinase* signaling network. Furthermore, ROS induce transcriptional changes show involvement of these molecules in regulating a plethora of biological processes at the transcript level. Therefore, ROS stand out as a good candidate for future research through classical as well as advanced bio-technological methods for a better understanding of plant biology.

Keywords
ROS · Defense signaling · Phytohormones · Metabolic pathway · Abiotic stress

21.1 Introduction

Deviations in the natural environment can lead to induction of stress in living organisms. This phenomenon has been widely observed in plants during abiotic stresses which include changes in temperature, pH of the soil, salinity and water content (Jaspers and Kangasjärvi 2010). Extremes in any of these conditions can lead to the development of reactive oxygen species (ROS) in the plant cells. Although ROS are generally produced inside plant cells as a by-product of the metabolic pathways, excessive production of these free radicals can lead to damage of the cell components due to oxidative stress (Sharma et al. 2012).

ROS can also act as signaling molecules, which recruit enzymes responsible for combating the oxidative stress in plants. Although the major understanding about the accumulation of ROS is related to their negative effects, evidence also suggests their positive role in various physiological processes. This is mainly achieved by means of activating the stress reduction pathways. The generation of ROS during abiotic stress results in signal transduction to regulatory circuits involved in combating the stress. There needs to be a balance between the generation of ROS and their scavenging by the ROS degrading enzymes. Plants have an efficient enzyme system, among which the *Superoxide dismutase* (SOD), *Glutathione peroxidase* (GPx) and *Catalase* (CAT) are responsible for degrading the unnecessary ROS (Mittler 2002). There are also certain non-enzymatic regulatory circuits in plants which deal with the scavenging of ROS to ensure that the plants do not suffer from damage due to ROS accumulation (Choudhury et al. 2013).

The exact pathway by which ROS leads to combating the abiotic stress is still unclear. However, it has been found that ROS results in the induction of expression of those genes which are involved in the regulation of stresses.There are evidences about the induction of the protein *kinase* (Liu et al.) pathways, which lead to the management of the redox status of the cells. The assimilation of stress in the plant cells is influenced by the action of the cellular pathways which are induced by ROS (Choudhury et al. 2013). Therefore, the ROS are important in terms of providing defense to the plants against the abiotic stress as illustrated in Fig. 21.1.

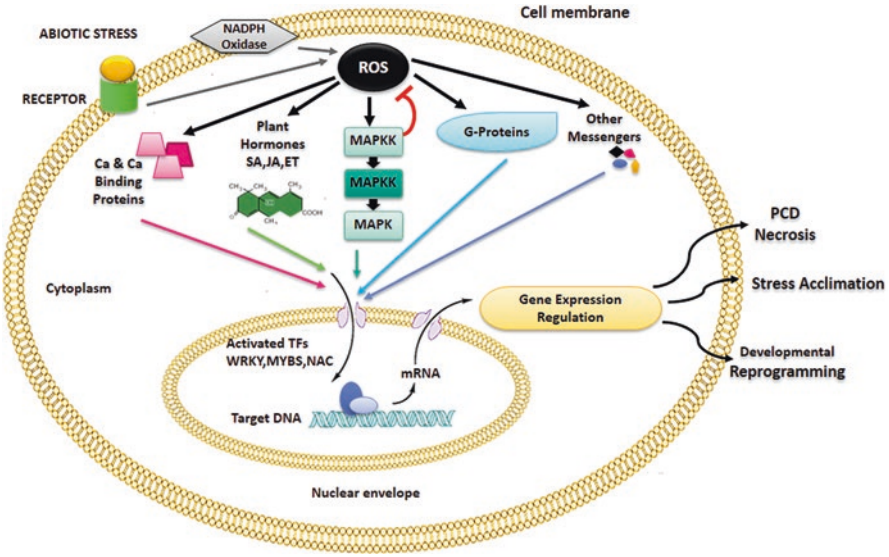

Fig. 21.1 Generalized scheme of abiotic stress induced ROS signaling in plants. Abiotic stresses trigger the production of intracellular ROS which act as versatile signaling molecules and modulate plant responses in numerous ways. They either interact with MAP kinases, secondary messengers or G- proteins and eventually activate transcription factors responsible for expression of genes, thus conferring abiotic stress tolerance

21.2 Production of ROS in Plants

The metabolic pathways operating under normal conditions result in the generation of ROS. During the electron transport in the process of respiration, photosynthesis and several other chemical reactions, ROS are produced as by-products. Various forms of ROS may include hydrogen peroxide (H_2O_2), hydroxyl radical (HO·), singular oxygen ($^1O_2$), and superoxide ion ($O_2{\cdot}^-$) (Das et al. 2015). Other means of ROS production include external stimuli or cell lignification processes which lead to the enzymatic production of ROS (Demidchik 2015). Recently, it has been found that ROS play an important role in cell signaling as they are important messengers for the initialization of redox reactions (Mittler et al. 2011; Schieber and Chandel 2014) and are involved in defense against external stresses and different developmental processes (Gapper and Dolan 2006; Baxter et al. 2013).

The major site of ROS production in plant cells is mitochondria, through the process of oxidative phosphorylation (Mignolet-Spruyt et al. 2016). The coupling of electron transport chain with oxidative phosphorylation results in the generation of ATP through electron transfer. This process is also mediated by electrochemical proton gradient that builds via proton pumping enzymes outlined in Table 21.1. During this electron transfer, there may be a leak of electrons, which can result in the reduction of molecular oxygen into the oxygen ion ($O_2{\bullet}^-$) followed by the development of OH and H_2O_2 (Blokhina and Fagerstedt 2010; Vanlerberghe 2013). One of the crucial steps for the production of oxygen radical in the RET pathway is the

Table 21.1 An overview of enzymes involved in production of ROS in different organelles

Production unit	Enzyme	Co-enzyme/ Co- factor	Reference
Mitochondria	NADH-Q oxidoreductase/ NADH dehydrogenase	Co-enzyme Q10/Ubiquinone and Quinone	Hirst (2005)
	Succinate-Q oxidoreductase/Succinate dehydrogenase	Flavin Adenine Dinucleotide	Cecchini (2003) Yankovskaya et al. (2003) Horsefield et al. (2004)
	Flavoprotein- Ubiquinone Oxidoreductase/ Flavoprotein dehydrogenase	Flavin Adenine Dinucleotide	Zhang et al. (2006b)
	Q-cytochrome c oxidoreductase/ Cytochrome bc1 complex	cytochrome c1 cytochrome b-562 cytochrome b-566 and a 2-Iron ferredoxin	Crofts (2004) Berry et al. (2000)
	Cytochrome c oxidase	Copper Magnesium Zinc	Yoshikawa et al. (2006) Tsukihara et al. (1996)
	ATP synthase		Yoshida et al. (2001)
Chloroplast			
	Plastocyanin	Chlorophyll Pheophytin Carotenoids Quinones	Jagannathan and Golbeck (2009)
	Cytochrome *b6f* complex (Plastoquinol-Plastocyanin reductase)	Iron sulfur cluster	Jagannathan and Golbeck (2009)
	Ferredoxin	Iron sulfur cluster	Jagannathan and Golbeck (2009)
Peroxisomes			
	Catalases Hydrogen peroxidase Flavin oxidases Glycolate oxidase	NADPH Flavin mononucleotide	Luis et al. (2006)
Apoplast			
	Peroxidases		Daudi et al. (2012)
	NADPH oxidases	Flavin Adenine Dinucleotide	O'Brien et al. (2012)
	Calcium dependent protein kinases	Calcium Magnesium Manganese	Baxter et al. (2013)
	CBL- interacting protein kinases	Manganese	Marino et al. (2012)

ubiquinone reduction (Blokhina and Fagerstedt 2010). In the case of ubiquinone being over reduced in the complex I, the RET activity can be reverted, causing the release of the oxygen radical inside the matrix of the mitochondria. If there is an over reduction at the complex III, the electron transfer to molecular oxygen cause the release of $O_2^{\bullet -}$ radical in the mitochondrial matrix and stroma.

Chloroplasts prove to be the site of constant production of the ROS in the plants in response to external stimuli, thus play an important role in ROS signaling. Hence, ROS are considered as important factors of cell signaling and communication by means of inducing changes in the gene expression in the nucleus (Lee et al. 2007b; Shapiguzov et al. 2012; Sierla et al. 2013). When the chloroplasts are exposed to abiotic stresses or intense lights, there is a decrease in the photosynthetic capacity which provokes the excess photon energy excitation that causes reduction of photosynthetic electron transport components (Dinakar et al. 2010; Adams et al. 2013) along with the production of ROS (Foyer and Shigeoka 2011; Fischer et al. 2013). There are three different ways in which the $O_2^{\bullet -}$ from the chloroplast can be produced via various enzymes summarized in Table 21.1.

In the optimum circumstances, the $O_2^{\bullet -}$ from the chloroplasts is turned into H_2O_2 through the SOD which are present in thylakoid and stroma. From here, oxidation leads to the production of water by the action of peroxidoredoxins and *Ascorbate peroxidases*. Under specific conditions, the excited P680 or PSII is the site of $^1O_2$ production. It causes the reduction of pheophytin and initializes a series of redox reactions through PET chain, forming reduced P700 along with the development of electrochemical proton gradient through the thylakoid membrane. However, in case of high photon energy or decreased rates of CO_2 assimilation, there is an excessive reduction of the PET components, which may cause an over-excitation of P680 and chlorophyll antennae. These in turn, manipulate or change the configuration of electrons in the oxygen molecules, giving rise to $^1O_2$ (Asada 2006; Fischer et al. 2013; Krieger-Liszkay et al. 2008), that can reciprocally react with the components present in the PSII and around it.

Another important site for ROS production is the peroxisomes. Various enzymes as stated in Table 21.1 (Luis et al. 2006) causes oxygenation of Rubisco in chloroplasts, subsequently leading to an initialization of the photorespiration pathway that results in H_2O_2 generation in peroxisomes (Kangasjärvi et al. 2012). This photorespiratory pathway proceeds with the production of glyoxylate from glycolate via *Glycolate oxidase* mediated oxidation, producing H_2O_2 (Bauwe et al. 2010). In C3 plants, this H_2O_2 production is a major oxidant source under light conditions (Noctor et al. 2002), as C3 plants have a tendency of increasing their photorespiration during stress, particularly at high temperatures (Sage 2013). During exposure to abiotic stresses, photorespiration is considered as the major source of ROS mediated signals (Voss et al. 2013; Weber and Bauwe 2013).

The continuation of the cell wall in the extracellular spaces leads to the formation of the apoplast. There are certain features like lower redox sensitivity of apoplastic proteins and lower buffering capacity of antioxidants have an impact on the redox properties of apoplast (Potters et al. 2009). Therefore, ROS accumulate in apoplast, initializing ROS mediated pathway, which ward off the low pH effects on the apoplastic protein's redox sensitivity and also affects the ascorbate gradient which ultimately leads to the homeostatic changes in the cell (Foyer et al. 2009; Munné-Bosch et al. 2013; Mignolet-Spruyt et al. 2016).

In times of abiotic or biotic stresses, the production of apoplastic ROS initiated by class III *peroxidases* and NADPH *oxidases*, is the first indication which can be

measured (Mignolet-Spruyt et al. 2016; O'Brien et al. 2012; Daudi et al. 2012). The reduction of the extracellular oxygen to $O_2^{\cdot -}$ is carried out by NADPH *oxidases* that require cytosolic NADPH as electron donor. These are the integral proteins of plasma membrane and are homologs of respiratory burst *oxidases* (RBOHs). The RBOH activation is caused by means of a conformational change in the EF-hand (Ogasawara et al. 2008) along with the phosphorylation of amino acids by different classes of *kinases* as described in Table 21.1 (Kadota et al. 2014).

The class III *peroxidases,* ROS producers of the apoplast work in many physiological processes and also during the defense responses against pathogens (Daudi et al. 2012; O'Brien et al. 2012). The mechanism of reaction of these peroxidases relies on the hydroxlic catalytic cycle and the peroxidative cycle (O'Brien et al. 2012; Lüthje et al. 2013). The peroxidative cycle is responsible for combining the reduction process of hydrogen peroxide to water with the oxidation of different substances, subsequently causing the production of $O_2^{\bullet -}$ (Liszkay et al. 2003). The hydroxlic catalytic cycle also mediates the reduction of hydrogen peroxide or oxygen radical ($O_2^{\bullet}$) (Passardi et al. 2004).

21.3 ROS and Stress Combination

When a plant faces two or more stresses simultaneously, the phenomenon is termed as stress combination (Mittler et al. 2006). Although it is explored recently at molecular level, it is considered as the major cause of crop loss worldwide (Mittler and Blumwald 2010; Rizhsky et al. 2004). As a general rule, in the presence of two or more stresses, the plants face higher negative impacts as compared to the effect of a single stressor. When two different stresses combine, such as heat and salinity or drought and heat, they trigger different types of transcriptomic responses that led to the generation of transcripts which are not produced if the single stress is applied (Rizhsky et al. 2004; Suzuki et al. 2016). In some cases, abiotic stresses like ozone give tolerance to plants against other biotic and abiotic stresses when two stresses are applied simultaneously (Mittler and Blumwald 2010; Suzuki et al. 2014; Foyer and Noctor 2016). In terms of involvement of ROS in stress, the levels of ROS and antioxidants as well as the activity of ROS scavenging enzymes have all been shown to have unique behavior during stress combinations. However, a differential pattern of each of these concepts is observed in case of application of individual stress. Such changes can be examined via the analysis of different parameters including the levels of H_2O_2 and $O_2^{\cdot -}$, the activity of enzymes including CAT, APX, SOD, AOX, *Glutathione reductase*, GPx, *Peroxidases* and *Glutathione-S-transferase,* the accumulation of various antioxidants (flavanols, alkaloids and carotenoids) and osmoprotectants (glycine betaine, sucrose and proline) (Jin et al. 2016; Carvalho et al. 2016; Martinez et al. 2016). It is quite possible that the generation of ROS under combination of different stress conditions is unique as diverse physiological responses are observed in plants. For example, two opposing responses are

demanded by the plant against combined effect of heat and drought stress: stomatal opening to reduce heat stress and closing of stomata to avoid the loss of water (Rizhsky et al. 2004).

In case of *Arabidopsis*, the cytosolic *APX1* mutants which had normal thylakoid *APX*, were seen to be sensitive to this combination of stress which suggests that the levels of cytosolic H_2O_2 is important and not the chloroplastic (Koussevitzky et al. 2008). The mutants which had impairments in the functions of ABA and PP2Cs showed sensitivity towards combinations of heat and drought and also the combination of heat and salinity. Various studies elucidated the roles of ROS in stress acclimation pathways as they are seen to elevate the levels of ROS specific transcripts during stress combination (Suzuki et al. 2014). Currently, the ABA and ROS are seen to be important for mediating the responses of plants against stress combination. In future, it is quite possible that studies would focus on the explanations about ROS roles in case of stress combinations.

21.4 ROS in Retrograde Signaling During Abiotic Stress

21.4.1 The Interplay Between ROS and Stress Hormones

Accumulation of ROS play important role in plant homeostasis by triggering various downstream redox signaling cascades, sugar sensing, ABA, Ca^{2+} fluxes and both ABA-dependent downs and upstream signaling pathways. Prolonged drought stress period leads to extensive cellular damage and even death because of enhanced activities of antioxidant.

Biosynthesis and catabolism activities of phytohormones like ABA play vital role in defense mechanism against abiotic stress and antioxidants. Du et al. (2010) isolated *dsm2* i.e. drought sensitive mutant rice, with impaired expression of *β-carotene hydroxylase* gene, that is involved in production of zeaxanthin i.e. carotenoid precursor of ABA.

In *dsm2* drought stress ultimately leads to non-photochemical quenching (NPQ) capacity, decrease in ABA, zeaxanthin and Fv/Fm level when compared to wild type. While overexpression will ultimately increase NPQ, expression of ABA associated genes and xanthophyll level which resulted into enhanced resistance against abiotic and drought stress (Du et al. 2010). *OsABA8ox3*, play a key role in accumulation of ABA and abiotic stress so, *OsABA8ox3* RNAi plants show better tolerance against drought stress, reduced level of MDA and increased CAT and SOD activities. In transgenic tobacco increased ABA content is reported due to the overexpression of the *9-Cis-Epoxycarotenoid dioxygenase* gene which ultimately leads enhanced tolerance against drought stress. In some studies it has been reported that abiotic stress tolerance is also associated with ABA dependent production of NO and H_2O_2 that further activate ROS scavenging enzymes (Zhang et al. 2009). Increased levels of ROS play key role, while H_2O_2 act as a secondary messenger, in specific signaling pathways that activate acclimatory/defense mechanisms.

21.4.2 Calcium/Systemic Signaling and ROS

It was found recently that ROS mediate the systemic signaling pathways in plants against abiotic stress responses by a process which couples electric waves and calcium signaling (Mittler et al. 2011; Gilroy et al. 2014, 2016). The health of a plant depends on the responsiveness of all its organs towards stresses in a coordinated manner. This coordination is believed to be dependent on the ROS production which is initiated in those plant cells that are affected directly by stress and then propagated as a wave through the whole plant at 8.4 cm/min rate (Miller et al. 2009). According to the ROS wave model, calcium influx into the cytosol in response to abiotic stress affecting local cells. This influx activates the *RBOHs* or initiates a series of events which, in turn, activate calcium regulated protein *kinases* that finally causes the phosphorylation of *RBOHs* (Miller et al. 2009, 2011; Gilroy et al. 2014). These phosphorylated *RBOHs* trigger ROS generation at the apoplast which is sensed by the nearby cells, leading to a flux of calcium in these cells followed by activation of their own *RBOHs*. The ROS dependent calcium influx together with *RBOHs* activated by calcium is transmitted throughout the whole plant and generated systemic responses to stresses (Miller et al. 2009).

It has been evident that ROS signaling in *Arabidopsis* is regulated by the *RBOHD* and is associated with a systemic generation of the calcium wave (Gilroy et al. 2016). ROS has been seen to be involved in the generation of electrical signals as a response to the abiotic stress. Also, the wave of ROS was seen to be involved in the systemic responses of plants towards heat or light stress (Suzuki et al. 2013). In terms of responding to heat, ROS coordinates with ABA in the systemic leaves (Suzuki et al. 2013). In the discovery of ROS wave (Miller et al. 2009) and their interactions with calcium wave (Gilroy et al. 2014), it was hypothesized that leaves respond to abiotic stresses in an autonomous way and these responses are associated with the functions of stomata, the response of one leaf being coordinated with that of the other via combined effect of ROS, calcium and hydraulics functioning together (León et al. 2001) with the electric waves (Mittler and Blumwald 2010). This proposed model was in line with the previously reported observations related to systemic stress responses in plants and also shows the role of ABA, ROS and stomata in the generation of plant systemic responses (Mittler and Blumwald 2010). Besides, *RBOHs* mediated regulation of ROS wave, several other ROS producers are also present as significant regulators of systemic signaling in plants. For example, in light stress responses, the production of singlet oxygen in chloroplast was found to be necessary in triggering the *RBOHs* dependent ROS wave (Carmody et al. 2016). Moreover, glutamate receptor like channels and nitric oxide have roles in eliciting systemic acquired acclimation via initiation of calcium flux, electric and ROS wave (Gilroy et al. 2016). These observations suggest the role and specificity of ROS wave in provoking systemic acquired acclimation in plants against abiotic stress responses (Suzuki et al. 2013).

21.4.3 Antioxidant Proteins

Detoxification of ROS is very important for protection against stress and various antioxidant enzymes are present for this sole purpose in all the cellular compartments of plants, and their role is also investigated in crop plants as well. Transgenic alfalfa when exposed to water deficiency starts expressing *MnSOD* cDNA that helps in improved survival rate and increased yield in natural fields over the time period of three winters (Mckersie et al. 1996). Improved tolerance against drought, oxidative stress and salinity were reported in transgenic plants, these were produced by transforming cDNA encoding a cytosolic copper-zinc SOD mangrove plant *Avicennia marina* into rice, when compared to wild type (Prashanth et al. 2008). Stress treatment in transgenic plants leads to Overexpression of *OsAPX2* which further decreases H_2O_2, MDA level and increases *APX* activity that ultimately leads to better tolerance against drought stress and under abiotic and cold stress increased fertility when compared with wild type plants (Sato et al. 2011).

During biotic and abiotic stress toxic products of ROS are accumulated with proteins and lipids that significantly damage the crop plants. In alfalfa better tolerance against drought and oxidative stress is reported due to the presence of NADPH-*dependent aldose/aldehyde reductase* which ultimately reduces toxic products resulted from lipid peroxidation and same results were also reported in tobacco plants that also express this *aldose/aldehyde reductase* (Oberschall et al. 2000).

21.4.4 Protein Kinases and Phosphatases

The *MAPK* (*Mitogen activated protein kinases*) pathways have a small amount of the *MAPK kinase kinase* (*MEKK/MAPKKK*) and *MAPK* along with the *MAPK kinase* (*MKK/MAPKK*). When *MAPKKK* is activated, a phosphorylation induced signal is processed downstream, which leads to the phosphorylation of *MAPKK* and finally leading to the activation of downstream MAPK (Hamel et al. 2006). These signaling pathways are highly conserved and have significant roles in many of the stress responses (Jalmi and Sinha 2015). They also play an important role in the signaling initiated by ROS. It has been proved through various studies that ROS do not only trigger but are also produced as the consequence of *MAPK* activated pathway (Pitzschke et al. 2009; Pitzschke and Hirt 2006).

21.5 ROS-Mediated Abiotic Stresses Acclimation/Mitigation

21.5.1 Role of ROS in Drought Stress

Plant productivity can be limited due to drought stress. As now a days global warming is a big issue worldwide which ultimately increase drought problem. Insufficient availability of water affects plants growth by interrupting the natural cycle of

photosynthesis. Environmental stresses directly affect the downstream genes and also on the enzymatic activity including CAT, POD and SOD and their expression ultimately leads to accumulation of ROS. To survive the drought stress there are various mechanisms that allow them to adapt such environmental conditions (Cruz de Carvalho 2008).

Low molecular weight proteins known as metallothioneins (MTs) having high Cys residues, play important role in metal binding, maintenance of redox level and ROS detoxification. In rice presence of type 1 MT, encoded by *OsMT1a*, as well as above mentioned anti-oxidant enzymes triggered by Zn^{+2} and dehydration ultimately leads to better accommodation of plants in stress environment (Yang et al. 2009).

Similarly in tobacco tolerance against salt and osmotic stress can be regulated by ROS scavenging through *sbMT2* gene (Chaturvedi et al. 2014). In Plants root tissues and cells start producing ROS when they are exposed to environmental stress like drought even for few minutes to hours that ultimately alarm all the cells and they in response start producing ROS. After initial phase of ROS outburst in second phase this oxidative burst spread to whole plant to improve the tolerance level that also includes downstream defense signal networks.

During drought stress changes in glutathione and ascorbate content of cellular compartment was studied for 10 days in glutathione deficient mutants' *vtc2-1* and *pad2-1*, ascorbate and *Arabidopsis thaliana Col-0* respectively (Koffler et al. 2014). In both mutants level of glutathione decreased when in leaves drought stress is not measureable yet. While in peroxisomes and chloroplast depletion of ascorbate was detected which can be explained and correlated with high level of H_2O_2 suggesting their role in drought stress by detoxification of ROS in vacuoles (Koffler et al. 2014).

21.5.2 Role of ROS in Salt Stress

Salt stress is one of the most important factors that limit crop production throughout the world. Salts through various osmotic and ionic effects cause medium to severe negative effects on plant productivity. Excessive salts in the soil solution causes inhibition of plant growth and ultimately death (Zhu 2001). The rate at which various salts are transported and accumulated in the leaves reaching toxic levels, determines the sensitivity or tolerance of different plants to salt stress. Salt tolerance is a highly complex trait achieved through control of water movement in roots and within the plant tissues and stomata, inter- and intra-cellular ionic balance, maintenance of antioxidant defense systems, and other functions potentiated by the coordinated action of several families of genes. The role of antioxidant defense system during plant response to salt stress is still largely unknown. The generation and scavenging of various types of ROS during salt stress responses carries huge importance, especially in relation to the different types of photosynthesis mechanisms i.e. C3, C4 and CAM (Abogadallah 2010). However, it has now become clear that maintenance of the overall cellular redox environment plays a key role in salt tolerance though the underlying signaling networks are highly complex involving several receptors and

transcription families. Recent research sheds light on the regulatory role of ROS during stress-triggered hormonal signaling such as abscisic acid, ethylene, salicylic acid and jasmonic acid signaling pathways (Golldack et al. 2014).

Excessive accumulation of salts in plant tissues impairs ionic and osmotic balance, photosynthesis and results in redox imbalance. Similarly, excess generation of ROS such as superoxide, H_2O_2, and NO cause oxidative damage of cellular membranes due to lipid peroxidation and interference in ion fluxes. However, current research emphasizes a dual function of ROS as a convergent point or signaling interface during plant responses to various abiotic stresses including salt stress and osmotic stress tolerance by sensing changes in the cellular redox state (Golldack et al. 2014). Perturbation in the expression of ROS-responsive *Arabidopsis WRKY15* abolishes the mitochondrial stress responses driving osmotic stress tolerance (Vanderauwera et al. 2012). The *Arabidopsis Haem oxygenases* isoform *HY1* plays a key role in photochrome chromophore biosynthesis. Loss of function *hy1* in *Arabidopsis* plants is sensitive to salt stress with no stress acclimation response whereas, plants over-expressing *HY1* exhibit salt tolerance. Though, mild salt stress increased in the expression of *RBOHD* and bi-phasic ROS production in WT plants, only the 1st peak of oxidative burst was observed in *hy1* mutant plants (Xie et al. 2011). This indicates that *RBOHD* derived ROS peak II is required for *HY1*-mediated salt acclimation. The small ubiquitin related modifiers (SUMO) are a group of proteins involved in post-translational modification (PTM) of various proteins, found in all eukaryotes. These PTMs are driven by SUMO activating, conjugating and *ligase* enzymes through the attachment of SUMO to target proteins. The role of these proteins in plant responses to biotic and abiotic stress responses has just been started to unravel. The *SaSce9* gene of the halophytic plant *Spartina alterniflora* is highly inducible by salt, drought, cold and ABA (Karan and Subudhi 2012) and encodes a 162 amino acid SUMO conjugating enzyme (SCE). This gene positively regulates salt stress tolerance as 35S::*SaSce9 Arabidopsis* plants show enhanced tolerance to salinity and drought and accumulated lower levels of ROS under saline conditions potentiated by high expression of antioxidant genes SOD, CAT and the ion antioporter genes *NHX1* and *SOS1*. Furthermore, these plants also showed enhanced expression of genes involved in ABA dependent signaling and Proline biosynthesis. Furthermore, the function of *SaSce9* appears to be highly conserved as ectopic expression of the *SaSce9* successfully complemented the SCE mutant *Ubc9* (Karan and Subudhi 2012). Salinity and osmotic stress have been shown to enhance the production of ROS resulting in oxidative damage (Zhu et al. 2007). Abiotic stresses including drought and salinity induce osmotic stress within the plant cells resulting in an acute imbalance of various solutes leading to loss of cell turgor and accumulation of various ROS. Salt stress induced by 100 mM sodium chloride (NaCl) treatment contributed to a 47% reduction in leaf fresh weight and 17% reduction in root length in sorghum plants indicating a severe negative effect on growth and development. At cellular level, this treatment decreased the relative water content of the roots by 23% with a concomitant 63% increase in the Proline content. At the same time, leaf H_2O_2 content was increased by 149% as compared to

sorghum plants treated with distilled water. Oxidative stress resulted in the oxidative degradation of lipids present in various membranes etc. as shown by 106% increase in lipid peroxidation ultimately resulting in increased cell death in NaCl-treated sorghum plants (Nxele et al. 2017).

21.5.3 Role of ROS in Heat Stress

Heat stress is constantly changing and damaging abiotic stress of the environment. In every decade 0.2 °C rise in worldwide temperature of air is predicted, consequently it may lead to comparatively 1.8–4.0 °C raise in temperature by twenty-first century than present time (Hasanuzzaman et al. 2013). Heat stress can cause devastating effects like oxidative stress, inhibition of seed germination, reduction of plant growth, improper development, and alteration in photosynthesis, alteration in phenology, water loss, yield reduction and reduction of crop quality (Hasanuzzaman et al. 2013).

Enzymes, sensitive to heat stress regulates some metabolic pathways, in which disentanglement of enzymes takes place due to heat stress resulting in collection of undesirable, destructive reactive oxygen species, cause of oxidative stress, includes $^1O_2$ (singlet oxygen), $O_2^{\cdot-}$ (superoxide radical), H_2O_2 (hydrogen peroxide) and $OH^{\cdot}$ (hydroxyl radical) mainly (Asada 2006; Hasanuzzaman et al. 2013). Sites for formation of reactive oxygen species are reaction centers of photosystem I and photosystem II present in chloroplast, peroxisomes, mitochondria (Soliman et al. 2011). Photosystems affected by heat stress will cause less absorption of photon (Halliwell 2006; Hasanuzzaman et al. 2013).

Heat stress is responsible for causing several physiological changes (Halliwell 2006; Hasanuzzaman et al. 2013). Hydroxyl radicals have capability to react with all components of cells example includes deoxyribonucleic acid, pigments, lipids and proteins (Møller et al. 2007; Karuppanapandian et al. 2011). Oxidation of poly un-saturated fatty acid, DNA and protein can be take place by Singlet oxygen (Huang and Xu 2008). Reactive oxygen species formation due to elevated electron seepage from thylakoid membrane cause oxidative stress, when heat stress influences photosynthetic light reaction (Halliwell 2006; Bavita et al. 2012). Heat stress causes the enhanced production of MDA (malondialdehyde), by reducing antioxidant enzyme action (Hurkman et al. 2009; Hasanuzzaman et al. 2013). Heat stress is inducer of oxidative stress and cause of damage of plants by enhancing membrane peroxidation and by decreasing the membrane thermostability by percentage of 28 and 54, resulting in elevated electrolyte escape from wheat (Miller et al. 2009; Hasanuzzaman et al. 2013). The comparison between moderate temperature, 36 °C and heat stress, 40 °C showed notable physiological destruction of utmost proficiency of photosystem II, destruction of cell membrane stability, lipid per-oxidation just due to production of large amount of hydrogen per oxide (H_2O_2) (Halliwell 2006; Hasanuzzaman et al. 2013). Heat stress triggered intense membrane wound and membrane lipid peroxidation is also reported in sorghum, soybean and cotton (Tan et al. 2011; Young et al. 2004; Hasanuzzaman et al. 2013; Mohammed and Tarpley 2010).

Reactive oxygen species formed due to heat stress are responsible for cotton's early leaf senescence. Biological function behind this is protein break down or formation of simple soluble shape of protein due to degeneration of polymeric proteins (Young et al. 2004; Hasanuzzaman et al. 2013). About 68% increase in production of ROS; such as superoxide anion ($O_2^{\cdot-}$) than normal level in root cells due to heat stress of just 2 days in wheat lead to obstruction of root growth, furthermore seedling development (2 days of exposure) is affected by enhanced MDA by percentage of 27, same inclination is tracked in late stages by percentage of 58 (Miller et al. 2009; Hasanuzzaman et al. 2013).

Reactive oxygen species gathered at the external surface of plasma membrane results in membrane depolarization due to heat stress, as well as ROS activates the calcium induced *RBOHD* enzyme, ultimately it can lead to programmed cell death (Qi et al. 2011; Hasanuzzaman et al. 2013). Plant metabolic pathways adversely affected by ROS in the presence of heat stress, need to develop heat tolerance plants are un-explainable and should be revealed by understanding the metabolic processes and signaling behaviors.

21.5.4 Role of ROS in Cold Stress

The Reactive Oxygen Species have a very important role in managing the abiotic stress in plants. There are multiple complex pathways which work together to reduce the load on the plant cells. ROS is among the central molecules of such pathway. Plants can face stress in the form of cold and drought. Under such conditions, the reactive oxygen can be generated in the cells to provide an indication of the stressed conditions. Although the production of ROS is known to create an oxidative stress in the cells, plants have evolved to survive such that they use the ROS as signaling molecules and indicators of stress. In the times of cold stress, the plant cells respond in a similar manner as to the other abiotic stress responses. It is important for the plants to be able to tolerate the cold stress. An inability to cope up with lower temperatures can lead to the yellowing of the plants. Moreover, the seed germination is also effected as the reproductive parts of the plants become damaged owing to cold stress. Cold stress can also lead to dehydration and hence lead to the drought conditions in the internal environment of the cells (Yadav 2010). Therefore, it is important for the plants to utilize the ROS mediated signaling pathways for developing tolerance towards the cold stress.

The major action in time of the abiotic stress is the production of the ROS in the cells. A minimal concentration of ROS leads to the signaling cascades inside the cells which cause the regulatory pathways to become activated. In the case of extreme conditions, there can be high levels of production of ROS, which may lead to programmed cell death (PCD) (Petrov et al. 2015). However, the development of plants has been such that they are able to tolerate a certain level of ROS and take it as a signal for stressed conditions. The overall mechanism of cold stress management involves the ROS as the core ingredients. The pathway starts with the recognition of cold stress and a disturbance in the metabolic functions. Once the metabolism

is disturbed, other disturbances begin to settle. Plants may also undergo dehydration and solute leakage from the cells. The production of ROS under such stressed conditions lead to the change in the transcriptional activities of the cells. The signal transduction of the regulatory pathways is hence triggered. As a result, the stress response genes are activated and they eventually begin to clear out the unwanted ROS. The toxicity of the cells is thus reduced and plants develop cold tolerance (Choudhury et al. 2017).

21.5.5 Role of ROS in Light Stress

Plants face different types of biotic and abiotic stresses. The light stress in terms of plants is an interesting phenomenon since they depend on light for growth and proper development. The dependency of plants on light for photosynthesis has caused the evolution of plants to be such that they intake a controlled frequency and wavelength of light. The overall growth and development of plants depends on their ability to sense light intensity and adjust to it accordingly. Any change in the light intensity or a deviation in the wavelength of the light can lead to a signal transduction from the chloroplasts. This is directly linked with the generation of the stress response. The ROS play an important role in such responses is generally the first signaling molecules to be produced as a result of stress to the plant cells. The light induced stress on plants can either be on the entire plant or on certain parts of the plants, for example, the roots. Since the roots grow away from the intensity of the light, an increase in the light can lead to stressed conditions for the roots and the growth of the plant can be effected (Yokawa et al. 2014). Hence, the tolerance mechanisms triggered by ROS are extremely crucial.

The ROS have been found to have a well-established role in almost all the cellular pathways of the plant cells. The plants have developed pathways which are dependent on the ROS. These pathways include cell signaling, plant growth and development and also the senescence of the flowers depends on signaling by the ROS (Bhattacharjee 2005). This is the point towards the importance of the ROS in maintaining the overall life cycle of the plants. In the case of light stress, plants produce ROS, which generate the redox signaling that trigger the tolerance mechanisms. The studies on the process of photosynthesis have proved to be very important in terms of understanding the ROS production and usage in the plants. The ROS have the tendency to interact between the different sub-cellular pathways. The chloroplasts and mitochondria are important in terms of understanding the processes of photosynthesis and respiration in the plant cells. When the light intensity deviates from the normal conditions, these organelles are seen to have an increased concentration of ROS which triggers the transcriptional activity of the stress response gene. The chloroplast develops tolerance to high intensity light as a result of the signaling by the ROS (Dietz et al. 2016). Therefore, the ROS are the most important species in terms of mediating plant responses against the light induces stress.

21.5.6 Role of ROS in High Frequency Electromagnetic Fields

High frequency electromagnetic fields (HF-EMF, i.e., frequencies ranging from 300 MHz to 3 GHz, having wavelengths from 1 m to 10 cm) are predominantly human-created, nonionizing electromagnetic radiations that don't normally happen to be found on the earth. HF-EMF is progressively present on earth (Balmori 2009) due to the dynamic improvement of remote innovation, including mobile phones, Wi-Fi, and different sorts of associated gadgets. HF-EMF acts as a strong stimulus that has the ability to initiate stress responses in plants similar to those triggered by other stress conditions.

Plants constitute an exceptional model to investigate their interactions with the external environment since their structure (high surface area to volume proportion) makes them more exposed to the surroundings. Moreover, plant species with favorable characters like ability to reproduce asexually and self-pollination can also be used to get genetically stable plant lines (Tkalec et al. 2007; Roux et al. 2006). Besides, species with altered metabolic functions constitute a valuable system to comprehend the way EMF (electromagnetic field) stimulus is transmitted (Beaubois et al. 2007). Plants respond to HF-EMF of even small amplitudes by inducing expression of genes or by altering their developmental organization (Halgamuge et al. 2015; Singh et al. 2012). Although the HF-EMF responses in plants need more explanations, yet they are considered as the major environmental constraint that initiates multiple responses in plants. One of these responses involves the alteration in the activity of enzymes that mediate ROS metabolism (Singh et al. 2012), a notable marker of plant reactions to different stress conditions. Similarly, EMF presentation stimulates specific molecular responses in plants against biotic stresses like wounding (Roux et al. 2006) and alters the progression of plants (Halgamuge et al. 2015). Besides, these reactions result in systemic acclimation as introduction of just a little locale of a plant brings about practically prompt response through the entire plant (Beaubois et al. 2007). These responses are negatively regulated by calcium chelators (Beaubois et al. 2007) or inhibitors of oxidative phosphorylation (Roux et al. 2008) which infers the inclusion of ATP pools.

Magnetic fields affect plants by increasing the production of ROS and uncoupling of free radical systems. Furthermore, the effects of MFs also include stimulation of ROS metabolism along with the modification of membranes via the formation of malondialdehyde (MDA, a notable marker of membrane alteration and oxidative stress) (Tkalec et al. 2007; Singh et al. 2012; Zare et al. 2015). Plants experiencing such alterations lead to the activation of signaling cascades that induce MFs specific responses. One of the key acclimation responses that plants undergo after they are exposed to EMF is the activation of ROS metabolism. Numerous plants exposed to high concentration magnetic fields experienced changes in the activity of antioxidant enzymes as well as accumulated ROS (Anand et al. 2012; Haghighat et al. 2014). Antioxidant enzymes, for example, *Peroxidase,* CAT, SOD and *Ascorbate peroxidase* have twofold to fourfold increment (Tkalec et al. 2007; Singh et al. 2012; Zare et al. 2015). The inquiry stays open to decide whether this could be the

result of an immediate activity of EMF on living tissue. Certainly, EMF at these frequencies changes into non ionizing radiations. Increased rate of ROS metabolism in response to EMF exert some damaging effects on plants like H_2O_2 formation, enhanced MDA production (Tkalec et al. 2007; Singh et al. 2012; Zare et al. 2015), and protein impairment (Radic et al. 2007).

In shallot leaves, apoplastic components are involved in regulation of redox signaling via sensing and responding to EMF stimulus. The consistent, low-power static MF (7 mT) and EF (20 kV/m) affect the antioxidant system of leaves, whereas EF alone induces lipid peroxidation and H_2O_2 production (Cakmak et al. 2012). In mung bean seedlings, the conductivity of electrolyte, H_2O_2 and O^{-2} levels, and malondialdehyde concentration decreased when the cadmium stressed seedlings were treated with 600 mT MF, whereas in case of cadmium stress alone, nitric oxide synthase (NOS) activity and NO concentration increased. It demonstrates that MF makes up for the toxicological impacts of cadmium stress linked to NO signal (Chen et al. 2011).

21.6 Various Roles of ROS in Plants

21.6.1 Role of ROS in Growth and Development

The Reactive Oxygen Species (ROS) have been identified to play a significant role in the growth and development of plants in addition to stress management. ROS generation and scavenging plays a significant role in the overall developmental processes of plants. The methods by which the ROS play a positive role in the plants include the cell signaling, cell to cell contact or through the cell wall cross linking. In most of these pathways, ROS act as an oxidant and facilitates the reactions which constitute the growth and development of the plants.

The cell cycle regulation in plants is carried out by means of a number of proteins. The growth and development phases require cell differentiation and formation of structurally diverse cells arising from the similar stem cells. This is where the role of the ROS has been found. In addition to their role in the stress responses, the ROS are also important in triggering cell differentiation and formation of the roots and shoots. The generation of H_2O_2 has been found to be particularly important for root elongation. However, in leaves a relatively low level of H_2O_2 is maintained for ensuring proper growth and expansion (Polyn et al. 2015). Hence, the role of ROS in the growth and development of plants is defined according to the organ in consideration. The cell differentiation process needs to be explored further to understand the exact pathway through which ROS mediated the differentiation and growth.

Cell cultures have further helped in identifying the events which lead to generation of ROS and their ultimate fate inside the plant cells. The concentration of ROS is reported to be non-uniform in the cell wall. The concentrations depend on the environmental conditions and their effects on the cell. During the growth phase, infection or pathogen invasion, highly transient peaks of the ROS are found in the cell wall. The control mechanisms of ROS are concerned with the maintenance of

the cytosolic pressure inside the cell. This is carried out by ejecting the H_2O_2 formed under stressed conditions. These pathways are also observed during the developmental phases of the plant cell as ROS mediates the cell wall thickening and loosening as per requirement (Kärkönen and Kuchitsu 2015).

21.6.2 Role of ROS in Stress Acclimation

Organisms living under aerobic conditions inevitably produce reactive oxygen species (ROS) as by-products of photosynthesis and respiration which are the most important energy producing processes in plants. With a plethora of various oxidase enzymes, mitochondria, peroxisomes, and chloroplasts are the main organelles where different ROS are produced (Apel and Hirt 2004). At the same time, there are mechanisms in place to utilize ROS in maintaining a balance between production and utilization. Plants in their natural ecosystem are always under stress due to a variety of biotic and/or abiotic factors. Through the billions of years of co-evolution, plants have adapted to cope with these adversities in many ways. Acclimation or acclimatization is a phenomenon through which plants adjust to the changes in biotic and abiotic factors around them, enabling them to maintain physiological activities at the optimum level. Technically speaking, acclimation happens within a relatively short period of time or within the organism's life compared to adaptation which is a developmental process that takes place through generations and is gradually incorporated into the genome over time. Therefore, acclimation is a more rapid or quick adjustment of morphological, physical and/or biochemical features of plants to the changing environment. Though, several plant species are known to acclimatize to changes in the environmental conditions around them, little is known about the underlying molecular mechanisms.

Reactive oxygen species (ROS) play an important role in acclimation of plants to abiotic stresses. By acting as systemic signaling molecules, ROS regulate different pathways during stress acclimation. As such, plant cells produce basal levels of ROS all the time. However, the type and quantity of these ROS may vary in different tissues as each type of cell/tissue has independent ROS-producing and ROS-scavenging machinery. Therefore, each of these sub-cellular compartments may have a variable ROS-signature at basal level (Choudhury et al. 2017). Furthermore, the quantity and type of ROS produced may also vary with the type of stress. Literature suggests that different abiotic stresses such as salt, heat, cold, high light, etc. may result in different ROS signatures. This indicates a high level of complexity as well as specificity in ROS-mediated stress responses (Choudhury et al. 2017). ROS also play a key role in acclimation of plants to stress combination as plants in their natural ecosystem, are exposed to a variety of abiotic stresses at the same time. However, it is also important to mention that various ROS once produced, are also toxic to the plants *per se,* therefore, there are various scavenging mechanisms in place for their detoxification. Several diseases in animals and humans are caused by oxidative stress which occurs due to an imbalance between ROS-producing and ROS-scavenging systems (Uttara et al. 2009). Oxidative stress in plants results in

the destruction of cells due to oxidative damage to membranes, proteins, RNA and DNA molecules (Mittler 2002). Thus, plants have developed a highly developed system consisting ROS-detoxifying proteins enzymes such as CAT, APX, SOD, GPx (Anjum et al. 2016; Gill et al. 2015) and other proteins e.g. glutathione, peroxiredoxin and ascorbic acid for regulating ROS levels, and to protect themselves from their toxic effects and use ROS as signaling molecules (Foyer and Noctor 2013; Noctor et al. 2017). Representative genes involved in ROS regulation and abiotic stress resistance are summarized in Table 21.2.

Plants adapt to stress conditions that happen on regular basis or more often such as drought, salt, and heat stress. Though the underlying mechanisms of these adaptations are highly complex and not well-understood, they involve programming of large cascades including various groups of proteins that function contemporaneously. For example, the heat shock proteins (HSPs) which are found in both animals and plants are encoded by a large and highly conserved group of heat stress-inducible genes. Heat stress results in elevated ROS levels in plant cells (Hasanuzzaman et al. 2012). Induction of HSPs has been shown to reduce oxidative stress (Oksala et al. 2014). Though plants cells contain efficient antioxidant machinery, changes in the cellular ROS-homeostasis do occur under stress (Foyer and Noctor 2013) which most often result in an increase in ROS levels. If this increase in ROS levels is relatively subtle, the in-house antioxidant machinery serves to reset the equilibrium between ROS production and scavenging processes. However, the increase in ROS

Table 21.2 Summary of important genes involved in ROS regulation and conferring resistance against abiotic stress

Functional gene	Plant specie	ROS adjustment	Abiotic stress resistance	Reference
sbMT2	*Nicotiana tabacum*	ROS scavenging	Salinity	Chaturvedi et al. (2014)
SaSce9	*Spartina alterniflora Arabidopsis thaliana*	ROS scavenging	Salinity	Karan and Subudhi (2012)
OsSRO1c	*Oryza sativa*	ROS accumulation	Drought stress,	You et al. (2012)
OsABA8ox3	*Oryza sativa*	ROS scavenging	Drought stress,	Cai et al. (2015)
ZmMPK5	*Nicotonia tobaccum*	ROS scavenging	Oxidative stress Salinity	Zhang et al. (2014a)
Ta-sro1	*Triticum aestivum Arabidopsis thaliana*	ROS accumulation	Salinity	Liu et al. (2014)
ZmMPK17	*Nicotonia tobaccum*	ROS scavenging	Cold stress Osmotic stress	Pan et al. (2012)
CAT,SOD	*Zea mays*	ROS scavenging	Salinity	Tseng et al. (2007)
PutAPX	*Puccinellia tenuiflora*	ROS scavenging	Salinity	Guan et al. (2015)
SIT1	*Oryza sativa*	ROS production	Salinity	Li et al. (2014)

accumulation is most often significantly higher under growth limiting conditions. Polle (2001) recorded approximately, threefold to tenfold increase in ROS production under stress conditions. Therefore, a transient oxidative burst and changes in the cellular redox state are common features of biotic and abiotic stress responses (Mittler et al. 2004).

21.6.3 ROS and Programmed Cell Death

As described earlier, different cellular compartments such as the apoplast, peroxisome, chloroplast, mitochondria, cytosol and the nucleus, have different basal and induced levels of ROS resulting in different ROS-signatures of various plant tissues (Choudhury et al. 2017). This enables the plant to regulate ROS-mediated responses in different plant cells independently during biotic and abiotic stresses. In this regard, programmed cell death (PCD) is perhaps the most prominent ROS-mediated phenomena in living organisms, which is the planned execution of only a specific type and/or number of cells.

In eukaryotes cell death happens most of the time through autophagy as part of the normal homeostasis when the old cells die and new ones are produced. In animals, apoptosis is a process homologous to PCD in which cellular suicide occurs through cell shrinkage and nuclear disruption followed by disruption of whole cells into fragments which are further processed through phagocytosis. However, plants neither have genes homologous to animal apoptosis-related genes nor they exhibit phagocytosis (Higashi et al. 2005). Autophagy is a major process for recycling of essential cellular organelles and ingredients such as proteins, and other metabolites and plays a key role in maintaining innate immunity and PCD (Kurusu and Kuchitsu 2017). PCD is a genetically regulated processes and evidence shows that it plays an important role in embryogenesis, xylogenesis, seed development/germination and other important developmental and processes (Kurusu et al. 2010; Bozhkov and Lam 2011) and in sexual reproduction as rice mutants defective in autophagy (*Osatg7-1* and *Ostatg9*) show sporophytic male sterility and immature pollen (Kurusu et al. 2014). This shows that PCD plays an important role in key physiological processes in plants. PCD occurs in response to biotic and abiotic stresses such as ozone (Langebartels et al. 2002), high temperature (Vacca et al. 2004), and lack or deficiency of oxygen (Jiang et al. 2010). However, cell death remains to be best described during hypersensitive response (HR) of the plants to infection by incompatible pathogens (Pennell and Lamb 1997).

In animal systems, the production of ROS such as nitric oxide (NO), hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot}$) are considered to be the hallmarks of cell death (Jabs 1999). However, the involvement of the above mentioned molecules during plant PCD remained obscure and only hypothetical for a long time. The inherent toxicity and short life span of these molecules also made it difficult to study their function during various physiological processes. Only recently, it has become evident that ROS production plays in important role in the regulation of developmental PCD such as the tapetal PCD that happens during pollen development in

plants. Rice mutant *mads3* exhibits abnormal tapetal PCD, abnormal ROS homeostasis in anthers (Hu et al. 2011). Production of ROS by the NADPH *oxidase* enzymes which are encoded by multiple homologues of the *Respiratory burst oxidase* (*Rboh*) is required for tapetal PCD and normal pollen development as *Arabidopsis* autophagy-deficient mutants such as *atg5* over-accumulate ROS (Yoshimoto et al. 2009).

The first evidence for the involvement of ROS in plant cell death came in 1994. Levine et al. (1994) showed a spatio-temporal correlation between ROS levels and plant cell death. Through studies involving cell suspension cultures they showed that ROS also act as signal during PCD in plants and that H_2O_2 mediated cell death could be inhibited by protease inhibitors and cyclohexamide. Since then, ROS have been considered as important molecules triggering PCD in plants. Later, studies involving the use of transgenic plants with perturbed levels of ROS due to overexpression and/or absence of cellular ROS producing and scavenging machinery, leading to abnormalities in ROS-driven PCD elegantly described the role of ROS in different events of PCD in plants (Mittler and Rizhsky 2000). ROS-driven PCD is characterized by a canonical bi-phasic oxidative burst (Van Breusegem and Dat 2006; Edreva et al. 2015). *Catalase* enzymes are the major scavengers of H_2O_2 in plant cells and different plant species have a variable number of genes encoding catalases. For example *Arabidopsis* has three well-known catalase genes which are expressed in different types of tissues and have different capacities for the decomposition of H_2O_2 and are variably activated by different growth limiting conditions (Du et al. 2008). *Catalases* deficient tobacco plants (Cat1AS) over accumulate H_2O_2 inducing cell death of parenchyma cells (Dat et al. 2003). Exposure to high-light for a short time induces accumulation of hydrogen peroxide which is sufficient to induce PCD in leaves whereas, long term exposure to high light resulted in continuous production of H_2O_2 causing necrosis and lipid peroxidation. This shows the existence of a ROS threshold below which PCD occurs and above which necrosis results in tissue death and degradation and inhibition of various component molecules required for PCD (Van Breusegem and Dat 2006; Kazzaz et al. 1996). Production of ROS such as singlet-oxygen (O_2) in the chloroplast of certain cells plays a key role in regulating the expression of nuclear localized genes through the *Executer1* (*EX1*) and *EX2* proteins localized in thylakoid membranes of the chloroplast, leading to a hyper-sensitive response (HR) to certain biotic and abiotic stimuli resulting in the death of those cells (Lee et al. 2007a; Kleine and Leister 2016).

Furthermore, various ROS have a variable capacity of initiating PCD suggesting a selective signaling effect of various ROS as they are interpreted differently at molecular level. For example, elevated ROS levels in *Arabidopsis lsd1* (lesion-stimulating disease1) and *rcd1* (radical-induced cell death1) mutants are sufficient as well as necessary for cell death induction (Mateo et al. 2004; Overmyer et al. 2005) whereas, *Arabidopsis flu* (conditional fluorescence) mutant generates singlet oxygen during circadian shifts, immediately initiating cell death response (op den Camp et al. 2003) suggesting a selective signaling effect of various ROS in plants (Danon et al. 2005). Similarly, *Arabidopsis vtc1* [vitamin C (L-ascorbic acid) deficient mutant] shows patches of dead cells on the leaves due to changes in redox

homeostasis rather than the quantity of ROS (Pavet et al. 2005). This shows that various ROS and changes in the cellular redox environment can activate separate/different signaling pathways that lead to PCD (Van Breusegem and Dat 2006) though the perception of different ROS signals at cellular level still needs to be investigated.

It has now become clear that various extracellular and intracellular cues integrate to trigger and regulate PCD or HR in plants. However, the recent burst in studies focusing on PCD in plants promises to provide important information about the succession of various events that lead to HR in plants. In this regard, several studies point toward the plant MAPKs as key converging points of various ROS triggers (Ren et al. 2006; Nakagami et al. 2005). Though, ROS-induced activation of MAP *kinases* indicate that the MAP kinase cascades work downstream of ROS (Nakagami et al. 2005). However, recent evidence suggests that these genes function upstream of the *RBOH* (Respiratory Burst Oxidase) genes which are responsible for ROS production during the 2nd burst of the biphasic ROS production in response to pathogen infection (Ren et al. 2006; Asai and Yoshioka 2008). Ectopic expression of the tobacco *KEK2* under the control of an inducible promoter is enough to activate other downstream kinases and cause cell death. Whereas, it's constitutive expression causes electrolyte leakage due to the loss of membrane potential and ROS production in chloroplasts and mitochondria. However, these plants failed to show any the cell death response when kept in dark due to failure in the accumulation of ROS in the plastid and *MEK2* activation, indicating the importance of chloroplast-generated ROS in *MAP-kinase* mediated cell death (Liu et al. 2007).

Based on studies, such as those conducted by (Joo et al. 2005; Liu et al. 2007) and (Zurbriggen et al. 2009) it is concluded that chloroplast is the initial source of ROS during the establishment of hypersensitive response leading to PCD. ROS-mediated signal transduction to the cell membrane potentiates an oxidative burst in the apoplast of concerned and surrounding cells and mitochondria though many steps of this workflow are still unknown and require investigative attention. Furthermore, it is interesting to mention that even if ROS production and/or homeostasis is abolished through mutations or generating transgenic plants, several aspects of the HR-PCD continuum remain un-affected indicating a strong contribution of ROS-independent processes in HR (Zurbriggen et al. 2009). Distinction of ROS-dependent and independent processes, their possible cross-talk with each other and contribution to PCD are attractive topics for future research.

21.7 ROS and MAPK Signaling Pathway Interplay

The sensing of ROS leads to the initializing of signaling pathway triggering the differential gene expression. ROS can lead to the activation of the signaling pathways in the cytoplasm or even the organelles in which it is produced. These are considered to be the moieties that activate the signaling pathways in a linear manner but there can also be instances of different patterns in these pathways (Jalmi and Sinha 2015). There is also a possibility that the pathways induced by ROS act to mediate

its production and keep a check on the overall concentration of ROS. When stress stimuli are encountered, there is an activation of the *MAPK* cascades. The *MAPK* are responsible for the activation of downstream pathways by means of phosphorylation and targeting of the transcription factors, phosphatases, kinases, cytoskeleton and its associated proteins (Hamel et al. 2006; Sinha et al. 2011).

In the case of environmental stimuli, the *MAPK* can work to regulate the ROS production activities (Asai et al. 2008). There are different *MAPK* cascades which are known to regulate the RBOH related oxidative burst and the development of disease resistance by ROS. These cascades include *MEK-2SIPK* cascade and *NPK1-MEK1-NTF6* cascade (Asai et al. 2008). The *MAPK* (*MEKK1-MKK5-MPK6*) pathway is involved in facilitating the expression of iron superoxide dismutase gene induced by salt and further leads to the production of ROS (Xing et al. 2015). These findings indicate that the action of ROS proceeds in a downstream manner from *MAPK* pathway.

The ROS is a crucial messenger which is generated in many stress responses and is known for affecting the *MAPKs* and resulting in upstream of *MAPK* pathway. When a pathogen is encountered, the ROS produced activated the cascade of *MAPK* (*MPK3, MPK4* and *MPK6*) in *Arabidopsis* plant. *MEKK1-MKK4/5-MPK3/6* cascade is activated by ROS in a defense response against pathogen (Asai et al. 2002). There is also another cascade of *MAPK* (*MEKK1-MKK2-MPK4/6*) which is known to be involved in working downstream of ROS in case of biotic as well as abiotic stress responses (Pitzschke et al. 2009). The *MAPK* are although activated by ROS but they are known to play a crucial role in the regulation of ROS production by means of a feedback mechanism. There are implications about the exertion of a positive feedback mechanism by *MAPK* cascade on the production of ROS. One particular study on maize showed that ABA causes the activation of MAPK which acts downstream of H_2O_2 and finally regulate the activity of RBOH positively for the production of H_2O_2 (Lin et al. 2009). *Oxidative signal-induced kinase 1* (*OXI1*) is a serine/threonine *MAPKKK* involves in *OXI1-MPK6* cascade which is responsible for the regulation of ROS production and it is activated by ROS itself (Asai et al. 2008). The *MKK4-MPK3/6* has a role in the production of ROS by working upstream of NADPH oxidase to H_2O_2 and is responsible for the activation of *MPK3 and MPK6* (Kovtun et al. 2000). In addition to the positive regulation of ROS production, the *NDPK2-MPK3/6* cascade has also been shown to play a negative role in the production of ROS during salt, oxidative and cold stress, thus resulting in the development of tolerance (Moon et al. 2003). This proves that ROS and MAPKs have an interconnected role in the maintenance of each other. However, the exact mechanism for the regulations remains to be explored.

21.7.1 ROS-Induced Transcriptional Changes

Through various genomic approaches hundreds and even thousands of genes involved in various interconnected signaling pathways and those that produce wide array of proteins like antioxidant enzymes, transcriptional factors and molecular

chaperones, have been identified that regulate stress responses in crop plants (Hu and Xiong 2014). Plant homeostasis involves various regulating networks and downstream processes that help them in balancing the level of ROS biosynthesis and ROS scavenging pathways. As it is stated previously that during abiotic stress level of ROS as well as antioxidant enzymes increased due to the action of hormones that are specific to environmental stresses.

ABA an important phytohormone utilizes various enzymatic and non-enzymatic constituents for protection against oxidative damage. As mentioned earlier that ABA accumulation resulted into enhanced production of ROS that ultimately trigger antioxidant pathways in crops while in rice NO, ROS, Ca^{2+} and calmodulin (CaM) play key role in ABA dependent antioxidant defense (Jiang and Zhang 2002a, b; Ye et al. 2011).

Moreover, in rice ABA induced expression of antioxidant enzymes (*SOD* and *CAT*), H_2O_2 that further activates *OsDMI3* and increased level of expression of *NADPH oxidase* genes depends on *Ca^{2+}/CaM-dependent protein kinase* (*CCaMK*) (Shi et al. 2012). According to this study ABA induced defense against anti-oxidative depends upon rice *Histidine kinase OsHK3* upstream of *OsMPK1* and *OsDMI3*. it is stated in some studies that *ZFP182, C2H2-type* and *ZFP* also play vital role in ABA dependent defense their expression is also prompted by ABA induced H_2O_2 and *OsMPKs* which are also known to up regulate the production of *MAPK* genes and NADPH (Zhang et al. 2014b).

In maize *ZmMPK5, CDPK* gene and *ZmCPK11* that acts upstream of *ZmMPK5* paly vital role in ABA-induced defense mechanisms and in return *ZmMPK5* also affect the productivity of H_2O_2 through NADPH *oxidase*, so, this whole process whole signaling process works like a positive feedback and consist of H_2O_2, NADPH *oxidase* and *ZmMPK5*. Furthermore, in maize H_2O_2 production is also associated with NO generation that leads to the activation of *MAPK* that further activates antioxidant enzymes (Zhang et al. 2006a; Hu et al. 2007; Ding et al. 2009; Lin et al. 2009). Hu et al. (2007) reported that in maize leaves up and downstream production of H_2O_2 also depends upon Ca^{2+}-CaM. While *Ca^{2+}/CaM-dependent protein kinase* and *ZmCCaMK* are also play essential role in ABA dependent defense. *ZmCCaMK* activations also depend on NO production that is induced by H_2O_2 (Ma et al. 2012).

In response to various biotic and abiotic stresses steroid hormones known as Brassinosteroids are also released that play vital role in developmental process of plants and their role in stress tolerance is also reported in various studies (Bajguz and Hayat 2009; Divi and Krishna 2009; Yang et al. 2011; Zhu et al. 2013). In maize *ZmMPK5* (that triggers NADPH oxidase-dependent expression of ROS) is also necessary for BR-dependent anti oxidative defense. Moreover *ZmMPK5* also involved in phosphorylation of ZmMAP65 and microtubule-associated protein (*MAP 65*) that in addition with some other molecules like, *ZmCCaMK, maize CCaMK* and Ca^{2+} play key role in BR- mediated antioxidant defense (Yan et al. 2015).

21.7.2 ROS and SRO Proteins

The protein family 'Similar to RCD ONE' (SRO) was identified recently as a plant specific proteins' group. They are characterized by plant specific domains: RST (RCD1-SRO-TAF4) domain at C terminal and Poly (ADP-ribose) polymerase catalytic (PARP) domain. The N terminal WWE domain is also present in some SRO proteins (Jaspers et al. 2010).

The knowledge about the SRO family is mainly from the studies on *rcd1* (*radical-induced cell death 1*), which is a mutant of *Arabidopsis.* The *rcd1* has influence on variable phenotypes which are related to developmental processes as well as to the responses against stimuli, including sensitivity towards salt and ROS, UV-B irradiation and tolerance to methyl viologen induced chloroplastic ROS (Ahlfors et al. 2004; Katiyar-Agarwal et al. 2006). The *RCDI* interacts with *SOS1* and many other transcription factors which are important in the stress responses and developmental processes (Katiyar-Agarwal et al. 2006; Jaspers et al. 2009). Recently, it is revealed that ROS induced gene expression might be regulated quantitatively via *RCD1* integrated signaling networks (Brosché et al. 2014). The *OsSRO1c* in rice was found to be a direct target for SNAC1, which is the drought related transcription factor (You et al. 2012). In guard cells, drought stress resulted in the increased expression of *OsSRO1c* that caused an accumulation of H_2O_2. This, in turn, led to decrease in the water loss and stomatal aperture. It was also found that the *OsSRO1c* had roles both in tolerance against oxidative stress and drought by means of accumulating H_2O_2 and inducing closure of stomata via DST regulators and SNAC1 (You et al. 2012). In wheat, *SRO* gene was recently reported to be involved in modulating the redox homeostasis, conferring resistance to salt stress (Liu et al. 2014). The salinity tolerant allele (*Ta-sro1*) in bread wheat is derived via point mutation from parent wheat. It has the PARP activity, which is in contrast to the SRO proteins of *Arabidopsis.* However, the expression of *Ta-sro1* in *Arabidopsis* and wheat regulates the activity of enzymes, resulting in ROS accumulation. *Ta-sro1* was also found to be responsible for activation of AsA-GSH and GPX cycle enzymes, which are involved in regulation of redox homeostasis and ROS content in cell (Liu et al. 2014).

21.8 ROS-Mediated Stress Tolerance: Prospects for Plant Biotechnology

The Reactive Oxygen Species (ROS) have long been a topic of great interest with reference to both plants and animals. The field of plant biotechnology still has a long way to go and the pathways triggered by ROS in terms of plant growth and stress responses are crucial in terms of understanding the ways in which the plant cells maintain their structure and functionality. ROS-mediated stress tolerance has been brought under question many times in order to understand the pathways in which plants manage cold, drought, salinity and other stresses. In each of these pathways, the production of ROS has been found to be central to the stress management and development of tolerance (Pérez-Clemente et al. 2013). The focus of the

newer biotechnological methods is now increasingly moving towards exploring the entire pathways through which the ROS mediate stress tolerance in plants.

The production of crops is one area of plant biotechnology which can greatly benefit by exploiting the ROS-mediated stress responses and tolerance in plants. Understanding how plants deal with the abiotic stresses such as high salinity can be useful in finding ways for managing the stress or the ROS pathway can be inducted and enhanced in plants growing under stressed conditions so that their growth does not retard due to a stressed environment. The processes of detoxification, stress management and hormonal responses to different situations all are somehow associated with the production of ROS in plants (Smékalová et al. 2014). Therefore, it is important to find out the metabolites and molecules which are triggered by ROS and the genes which are regulated as per requirement under the stressed conditions.

The emergence of newer molecular techniques and the wide spread use of the genomics, transcriptomics, and proteomics can be employed for gaining an understanding about the ways in which plants manage stress through ROS. This is of great interest since there is a delicate balance between the signaling function and the damaging functions of the ROS. If the ROS concentrations are beyond a certain threshold, they can cause cell death. Hence, in order to understand plant growth and response to stressed conditions, there is a need to explore the role of ROS in the different molecular pathways which occur inside the plant cells. One of the major implications for the field of plant biotechnology is to understand and exploit the ROS mediated stress tolerance for the development of crops that can withstand the harsh environmental conditions (Ahanger et al. 2017). Hence, the future of the agricultural field can be very bright if plant biotechnology advances and explores the role of ROS in depth.

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Plant Viruses as Virus Induced Gene Silencing (VIGS) Vectors

22

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Abstract

Virus-induced gene silencing (VIGS) is widely used to analyse the gene functions in model plants and in the plant species where generation of stable genetic transformants to downregulate gene expression is laborious and time-consuming. Plant viruses serve as a suitable candidate for understanding functional genomics by their modification as Virus Induced Gene Silencing vectors. Recent advancements in genetic engineering tools have made a significant contribution to their use as vectors. Here in this chapter, we have tried to discuss about the use of various plant viruses as gene silencing vectors and the next-generation vectors.

Keywords

RNA interference · RNA virus · RNA silencing · CaMV35S

22.1 Introduction

Viruses cause numerable diseases in plants as well as in animals. They have DNA or RNA as their genetic material with a protein coat protecting it. The majority of the viruses infecting plants have RNA as their genetic material. They have simple

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mode of replication and virus completes its life cycle after the viral RNA translates itself and complete formation of viral particle occurs (Hull 2008). Virus utilizes host machinery for its survival and can trigger number of reactions for their movement and replication inside, leading to an array of disease symptoms by hampering plant physiology.

With the recent significant advancements, genome sequencing through NGS and cloning full-length viruses with diverse genetic engineering tools has become much easier. Generating infectious clones of these viruses and using them for gene silencing has now become a choice for functional genomics. VIGS is a reverse genetic strategy that can allow us to functionally annotate the genes. VIGS utilizes an RNA-mediated antiviral defence mechanism of host which silences virus encoded transcripts. The technology has been exploited with viruses amenable of carrying gene sequences that are identical to the endogenous host target gene/s to silence them and deciphering their function. The viral double-stranded RNA (dsRNA) formed during the replication of viral RNAis identified by plant's defence machinery and the DICER proteins come into play to degrade them into small-interfering RNAs (siRNAs), which further leads to down-regulate the gene of interest via this mechanism. VIGS has been successfully used in cases where genetic transformation was not successful. Plant viruses serve an important VIGS vectors because they can lead to systemic infections which makes the functional annotation of genes easier. Large number of viruses have been developed and now extensively used for functional genomics. VIGS vectors are used for studying gene functions in tomato, pepper, soybean, barley, potato, pear etc. (Liu et al. 2002; Wang et al. 2013; Zhang et al. 2013; Scofield et al. 2005; Faivre-Rampant et al. 2008; Sasaki et al. 2011).

VIGS provide a most suitable way of transformation of the plant transiently. It has many advantages over any other known methods of transformation. It's low cost and more robustness adds to its quality.

22.2 VIGS Technology

VIGS technology is entirely based upon the backbone of the infectious viruses clones modified using one or more mutations/modifications. There are certain properties of the virus to serve as candidate to be developed into VIGS vector. The candidate virus must have a broader host range, should amount infection with good virus titre and without obvious symptoms are few among the list. The infectious clones of the Virus genome are primary requirement to develop VIGS vectors. There are two popular methods to construct infectious clones: (1) constructing full length cDNA clone of virus and obtaining RNA transcripts from it under the influence of a bacteriophage RNA polymerase promoters like T7, T3 or SP6, and (2) The expression of infectious viral RNA's by *in vivo* transcription of cDNA-containing vectors through a constitutive CaMV35S promoter. There are many viral infectious clones developed and used using these two techniques (Table 22.1). In later case, many steps of transcription can be avoided. This is particularly important for RNA viruses for which the production of good yield transcripts can be problematic. It is quite

Table 22.1 List of viruses with infectious clones developed using the two strategies

Family	Genus	Species	Strategy used
		Pepino mosaic virus (PePMV)	Both[a]
AlphaFlexiviridae	*Potexvirus*		
		Potato virus X (PVX)	Both
	Capillovirus	*Citrus tatter leaf virus* (CTLV)	Infectious transcripts
BetaFlexiviridae	*Citrivirus*	*Citrus leaf blotch virus* (CLBV)	Both
	Vitivirus	*Grapevine virus A* (GVA)	Agroinfection
Closteroviridae	*Closterovirus*	*Citrus tristeza virus* (CTV)	Both
	Macluravirus	*Chinese yam necrotic mosaic virus* (CYNMV)	Agroinfection
Potyviridae	*Potyvirus*	*Potato virus Y* (PVY)	Infectious transcripts
	Furovirus	*Beet necrotic yellow vein virus* (BNYVV)	Agroinfection
		Ribgrass mosaic virus (RMV)	Agroinfection
Virgaviridae	*Tobamovirus*	*Tobacco rattle virus* (TRV)	Agroinfection
		Tobacco mosaic virus (TMV)	Agroinfection
Secoviridae	*Comovirus*	*Radish mosaic virus* (RaMV)	Agroinfection
	Cheravirus	*Apple latent spherical virus* (ALSV)	Agroinfection

[a]Both agroinfection and viral RNA transcripts

inexpensive as no RNA cap analogs or RNA polymerases are required. VIGS utilizes the plant virus genomes along with the target genes are transformed via Agrobacterium. Viral DNA under the influence of CaMV35S promoter transcribes into RNA which then gets replicated via host RNA dependent RNA polymerase enzyme leading to an event in which double stranded RNA (dsRNAs) formation occurs and which ultimately triggers RNA silencing machinery. These dsRNAs are cleaved to small interfering RNAs by the activity of DICER-like proteins (DCLs). These siRNAs are recruited by the RNA induced silencing complex (RISC), turning them into single-stranded RNAs. These single-stranded RNAs search for complementary RNA sequences of the host which upon complementation are degraded. These developed infectious clones are used to carry foreign gene inserts resembling the endogenous host gene targets which can then be silenced through RNA silencing. The first RNA virus to be used as VIGS vector was *Tobacco mosaic virus* (TMV; Kumagai et al. 1995). The simplest way of inoculating the virus on the test plants makes virus a subtle choice to be used as gene silencing vectors. *Phytoene desaturase* gene was silenced using TMV based VIGS vector in *Nicotiana benthamiana* by cloning of partial cDNA encoding PDS gene in viral vector(Kumagai et al. 1995), 1 week after inoculation systemically infected *N. benthamiana* plants. This work was first of its kind demonstrated that a viral vector can be used to silence any target gene. *Tobacco rattle virus* based vectors have been efficiently used to silence genes in *N. benthamiana* and Tomato as well and the modified version is now the choice for its use to modify model plant *Arabidopsis thaliana* (Liu et al. 2002; Turnage et al. 2002).

Diversified viruses have served as candidate because of certain viral properties of which small size of the virus, broader host range, absence of severe infection symptoms and its infectivity matters the most. Now there are ranges of viral vectors which have been developed for different crop systems. We are covering several important viruses with the technologies that have been used to develop them, their uses and future applications.

22.3 Viruses Modified in to VIGS

Over the past few years, many vectors have been developed for use in VIGS, which includes *Tobacco Mosaic Virus* (TMV), *Potato virus X* (PVX), *Tomato golden mosaic virus* (TGMV), *Tobacco rattle virus* (TRV), satellite virus-induced silencing system (SVISS), *Barley stripe mosaic virus* (BSMV), *Apple latent spherical virus* (ALSV) and *Cabbage leaf curl virus* (CbLCV). Selection of viral species efficiently working as VIGS vector was based upon the host they can infect. Viruses with broader host range enabled the use of them as VIGS vector for many plant species.

Tobacco Mosaic Virus (TMV) The use of *Tobacco mosaic virus* as VIGS vector for silencing ChIHgene (Magnesium- chelatase gene) in *N. benthamiana* plants is one example of VIGS vectors. The gene which encoded the H-subunit of the magnesium-chelatase enzyme was silenced in the apical tissues, where suppression of both the TMV vector construct and the ChlH mRNA results in a strong reduction of the virus vector in the shoot apex. Lack of white and yellow phenotypes was initially observed on the growing apex, young leaves and stems, forming mosaic tissues thereby exhibiting reduced amount of ChIH mRNA in the affected tissue. Consequently, the plant apex partially recovered from the silenced phenotype leading to the increase in levels of both the ChlH mRNA and the TMV RNA and therefore, the silenced phenotype was re-established. Although the fluctuation of the silenced and recovered phenotypes appeared to be regulated in a feedback loop by a reduction and increase of the target mRNA and viral RNA levels, it also indicated that the TMV VIGS-mediated silencing was not systemically spread in the plants. It was observed that although TMV-ChlH–mediated silencing led to strong suppression of the virus vector, it did not lead to stable recovery of the plant from virus infection or from the VIGS-mediated silencing of the target gene (Hiriart et al. 2003)

Potato Virus X (PVX) PVX based VIGS vector is another example which triggered a VIGS response in both diploid and cultivated tetraploid Solanum species. The PVX construct with GFP insert was used to investigate the infectivity of the virus in these species. Both infiltrated and systemic upper-uninoculated leaves were harvested after agro-inoculation and the efficiency of gene silencing by binary PVX vector was assessed using RT-PCR by its ability to silence an endogenous *pds* gene in these species. Down-regulation of *pds* gene expression resulting in a characteristic photobleaching phenotype exhibits gene silencing. Photobleaching was observed

on all *N. benthamiana* plants by 12–15 days post inoculation when the cDNA region was sub-cloned in antisense orientation into the PVX vector, which is an indicative of *pds* silencing. The VIGS-based approach was also studied in *in vitro* grown potato species by assessing the down regulation of *pds* in *S. tuberosum* L. cvs Desiree micro propagated plants. Reproducible PVX infections were generated after the leaves of these plants were stab-agro inoculated with plated agrobacteria transformed with either PVX-GFP or PVX-PDSAS (pds anti-sense) constructs. After 4 weeks of inoculation, photobleaching phenotype was observed on systemic areas of leaves, indicating that silencing of endogenous genes (*pds*)in potato can potentially extend through the whole plant, including tubers. Although this vector is more stable than the TMV-based vector, PVX has a more limited host range than TMV, with only three plant families having members that are susceptible to PVX infection. Also, both TMV and PVX-based vectors can cause viral disease symptoms on inoculated plants thereby interfering with gene silencing effects (Ratcliff et al. 2001).

Apple Latent Spherical Virus (ALSV) The use of ALSV as VIGS vector has overcome the limitations of viral symptoms which often interfere the silencing of target genes as seen mostly in soybean (*Glycine max*). The viral symptoms because of some VIGS vector include necrosis, chlorosis and leaf distortion along with limited movement within the leaf tissue causing uneven phenotypes. ALSV, isolated from apple (*Malus pumila*) in Japan, has recently emerged as an efficient candidate for reverse-genetic tool of VIGS (Kasajima et al. 2017). The ALSV cDNA was mobilized into a binary vector compatible with *Agrobacterium tumefaciens*-mediated delivery into *Nicotiana benthamiana* leaves by the process of agro-infiltration. This inoculation process upon modification by using infected *N. benthamiana* homogenate to directly rub-inoculate the first unifoliate of young soybean seedlings have proved to be an improved method as it bypassed the need for particle bombardment along with rapid propagation of inoculum (5–10 days). The result was evaluated in 19 soybean genotypes among which photo-bleaching indicative of *Phytoene desaturase gene* (GmPDS1), silencing was observed in nine and two of them exhibiting photobleaching in 100% of the inoculated individuals (Gedling et al. 2018). The virus is serving as a very good candidate as by now the infection of the virus can be eliminated from infected plants by thermotherapy.

Sugarcane Mosaic Virus (SCMV) SCMV based VIGS vector is another potential vector to knock down the expression of endogenous and exogenous genes in *Nicotiana benthamiana*. In the experiment conducted by Ali et al. (2017), SCMV-VIGS vector construct was developed by replacing the RNA 1 and the two non-structural proteins of the RNA 2 with SCMV CP gene segment. This construct was cloned into an agrobacterium-mediated binary vector and were transformed by electroporation into *Agrobacterium tumefaciens* strain. Inoculation of SCMV based VIGS construct in *N. benthamiana* leaves was done using a needleless syringe

above the cotyledon. Semi-quantitative RT-PCR analysis was done to evaluate the level of the target gene. Successful post-transcriptional gene-silencing (PTGS) of the target genes, GFP and ChlI was observed. The ChlI gene is responsible for carotenoid biosynthesis when silenced causes photobleaching and characteristic loss of chlorophyll as a result of reduced carotenoid level and initiation of photo-oxidation. The inoculated plants exhibited more than 95% reduction in the transcript levels which confirmed the capability of this vector of endogenous gene silencing. Also, silencing of GFP causes phenotypic changes in the plants resulting in a change of colour from green to red under UV illumination due to chlorophyll autofluorescence. The inoculated plants were observed to successfully maintain the gene silencing for a couple of years as well as in the next progeny. Hence, SCMV-VIGS vector with its ability to exhibit visual phenotypic changes makes it a potential candidate for systemic gene silencing in plants.

Barley Stripe Mosaic Virus (BSMV) Until the 90s, VIGS was limited to dicot plant species. But with the advancement in the studies of genetic engineering and plant virology, gene silencing could also be achieved even in monocots. One such example was the use of BSMV based VIGS vector to knock down the expression of *Phytoene desaturase* endogenous gene (PDS) in barley, a monocot host. Holzberg et al. (2002) designed the BSMV based vector construct to express a *pds* fragment in both the presence and absence of coat protein. The constructs having three RNA transcripts (RNA a, RNA b and GFP-anti sense) were inoculated in barley pants. The spread of virus was monitored at regular intervals post inoculation. The appearance of mosaic symptoms progressed between 8 and 10 days where the barley leaves showed white streaks and patches lacking necrosis. The degree of photobleaching exhibited by the plants was assessed to evaluate the gene silencing by this vector construct. Holzberg and his team also inoculated the BSMV-bPDS construct in four different plant species (*Hordeum vulgare, Oryzae sativa, Zea mays* and *Nicotiana benthamiana*), which has its coat protein deleted. On regular monitoring of the effects of inoculation, it was observed that the infected plants showed consistent photo-bleaching, except for *N. benthamiana* suggesting that coat protein deletion caused a significant gene silencing of the PDS endogenous gene. The nPDS fragment expressed by the viruses exhibited neither visual change in phenotype nor any accumulation of phytoene, indicating that *N. benthamiana* having the least homology to barley species cannot silence barley PDS. BSMV-based VIGS vector could be a powerful tool to study gene silencing in monocot species.

Tobacco Rattle Virus (TRV) TRV uses two vector systems both of which are required for infection. Ratcliff F. and his teammates in 2001 assessed the potential of TRV to silence gene by infiltrating 3-weeks-old *N. benthamiana* leaves with agrobacterium pBINTRA6 and pTV00 using needleless syringe. The accumulation of virus RNA was monitored regularly post inoculation. The affected tissues such as leaves, stems, shoots and roots showed loss of green fluorescence and exhibited red

phenotypic change under UV illumination, suggesting the silencing of GFP gene. TRV has an upper advantage from other viruses such as PVX as the effect of gene silencing is persistent affecting more number of tissues with greater reduction in accumulation of mRNA. Although TRV is able to infect growing points and the spread of infection is more uniform by cell division and transport, this vector induces very mild symptoms, making it a potential VIGS vector for gene silencing (Valentine et al. 2004). TRV has been successfully used as VIGS vector in *N. benthamiana*, tomato, chilli pepper and rose (Senthil-Kumar et al. 2007; Li et al. 2013; Choi and Hwang 2012; Dai et al. 2012).

Tobacco Ringspot Virus (TRSV) Recently, TRSV-based vectors were developed for efficient VIGS in *N. benthamiana, A. thaliana*, legumes and cucurbits. In the first advancement, the GFP gene was inserted between the movement protein and coat protein region at MP-CP cleavage site for release of GFP. Efficient VIGS of phytoene desaturase (PDS) in plants demonstrated the use of TRSV as VIGS vector (Zhao et al. 2016).

22.4 Next-Generation Vectors and Limitations

The current VIGS approach and system has undergone several improvements for functional characterization of the host genes. The current system involving two RNAs of TRV for successful infection was reduced to single RNA (RNA1) component of the virus by removing 16 K cysteine rich protein (Deng et al. 2013). The protein has role in pathogenicity and removal of which created space for cloning new genes to be silenced. Some VIGS vectors have now been developed to target transcriptional level of silencing the gene for which the endogenous targeted gene promoters are cloned and expressed via VIGS which facilitates RNA directed DNA methylation resulting in gene silencing (Kanazawa et al. 2011). A modified virus vector has been developed for the expression of artificial micro RNAs (miRNAs) in plants (Tang et al. 2010).

VIGS utilizes the post-transcriptional gene silencing (PTGS) machinery of host plants to down-regulated the expression of the targeted gene by degrading the targeted plant gene expression along with the viral RNAs. However, the complete elimination of the transcripts of desired gene is not achievable by this method. Therefore, attempts were also made to develop an easy and efficient technique that causes silencing of the targeted gene and they can be good replacement of the VIGS vectors. DNA viruses such as bean yellow dwarf virus (BYDV); wheat dwarf virus (WDV) and cabbage leaf curl virus (CaLCV) and RNA virus as tobacco rattle virus (TRV), cucumber mosaic virus (CMV) or tobacco mosaic virus (TMV) have been modified successfully in transient gRNA delivery vectors and demonstrated efficient gene targeting *N.benthamiana*, potato, tomato, rice, and wheat (Zaidi and Mansoor 2017). Yin et al. (2015) has developed a Cabbage leaf curl virus (CaLCuV)-based guide RNA delivery system for CRISPR/Cas9 mediated plant genome editing

(VIGE) and demonstrated the powerful silencing of NbPDS3 and NbIspH genes which cause photo-bleached phenotype on leaves. These advancements in techniques offered an excellent chance to directly manipulate plant genomes. With recent advancements in the VIGS approach, the deliverance of the viral vectors still remains as a challenge and needs further improvements.

22.5 Conclusions

With the advancements in NGS technologies, genome sequencing has become much easier. Many genome databases of different plant species are now available. But the sequences remain as text until they have a function annotated to them. With large sized eukaryotic genomes, the functional annotation is a cumbersome and laborious task. With the development of genetic engineering tools, things have become much easier via development of these VIGS vectors. For instance, earlier amplifying and cloning the complete genome of viruses in an instance was difficult. But with the availability of advanced *Taq* and cloning tools, things have become much easier.

Different plant viruses have been utilized as VIGS vector for different plant species they can infect. The versatility of the VIGS based vectors lie in the fact that now many different plant species which do not serve as host for virus naturally, can be infected by viral VIGS vectors. VIGS approach is a promising tool and will be used widely. The concern regarding certain viruses which do not have infections in different parts of the world can be overcome by developing more virus based VIGS vectors. Virus based vectors have not only been used to silence endogenous genes but also as expression vectors which will be discussed elsewhere.

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Post-transcriptional Gene Silencing as a Tool for Controlling Viruses in Plants

23

Nikolay M. Petrov, Mariya I. Stoyanova, and R. K. Gaur

Abstract

RNA gene silencing is a mechanism for gene regulation, which limits transcription level by suppression of transcription (transcriptional gene silencing, TGS) or by activation of a process of degradation of specific RNA sequence (post-transcriptional gene silencing, PTGS), known also as RNA interference (RNAi). RNA interference was observed for the first time by chance during 1990, when in an attempt for over-expression of the chalcone synthase gene in petunias by insertion of its chimeric duplicate, the result was just the opposite – blocking of anthocyanin biosynthesis. After 16 years of experiments, the significance of this phenomenon has grown so much, that a Nobel Prize was awarded to Fire and Mello for its discovery. This chapter presents a retrospection of RNA gene silencing, its mechanism of action, corresponding participants, role in plants, and possible applications with a focus on the perspectives for utilizing this mechanism as a tool for control of viruses in plants.

Keywords

RNA interference · RNA gene silencing · Post-transcriptional gene silencing · PTGS · RNAi · Classes of RNAs · Virus control

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23.1 Historical Retrospection

RNA gene silencing is a mechanism for gene regulation, which limits transcription level by suppression of transcription (transcriptional gene silencing, TGS) or by activation of a process of degradation of specific RNA sequence (post-transcriptional gene silencing, PTGS), also known as RNA interference (RNAi).

Prior to the discovery of gene silencing, scientists have applied various methods to generate specific loss-of-function such as introducing T-DNA elements, transposons, treatment with mutagens, irradiation, and antisense RNA suppression. These methods are both time-consuming and not always working satisfactorily. Transposons and T-DNA elements sometimes accidentally incorporate into the genome, resulting in highly variable gene expression. Antisense RNA technology was found in plants when the inhibition of nopaline synthase gene was observed in tobacco cells, due to the expression of its corresponding antisense RNA (Rothstein et al. 1987).

RNA gene silencing was observed for the first time by chance during the distant 1990 in an experiment for over-expression of the chalcone synthase gene (*CHS*) in petunias by insertion of a chimeric *CHS* duplicate (Napoli et al. 1990). Instead, the experiment resulted in blocking of anthocyanin biosynthesis and production of white or pale patterned flowers (Napoli et al. 1990). Analysis of RNAs showed a 10 to 50-fold decrease of the level of mRNA produced by endogenous *CHS* compared to wild types, although there was no change in the developmental timing of mRNA expression (Napoli et al. 1990). Three years after the observation of this phenomenon Lee et al. found out that the heterochronic gene *lin*-4 of the nematode *Caenorhabditis elegans* does not encode a protein, but produces two small RNAs of approximately 22 and 61 nt with complementarity to a repeated sequence element in *lin-14* mRNA. Thus, *lin-14* translation is regulated by *lin-4* via an antisense RNA-RNA interaction (Lee et al. 1993). In 1995, Guo and Kemphues introduced RNA in *C. elegans* in attempt to block the expression of mRNA of the *par*-1 gene. However, they found that the par-1 mRNA itself also repressed *par*-1.This paradox observing, later named as RNA interference (RNAi) inspired the experiment of Fire et al. (1998), who described the effect of gene silencing caused by double-stranded RNA (dsRNA) in *C. elegans* by injection of dsRNA, complementary to *unc22* gene, responsible for the body morphology (Fire et al. 1998). Fire et al. (1998) also proved that the small interfering RNA (siRNA) is the initiating molecule to induce gene silencing in *C. elegans.* For their discovery of RNA interference – gene silencing by double-stranded RNA, Andrew Fire and Craig Mello were awarded the Nobel Prize in Physiology and Medicine in 2006 (https://www.nobelprize.org/nobel_prizes/medicine/laureates/2006/). siRNA was also discovered to be a part of PTGS in plants (Hamilton and Baulcombe 1999). Not much later, in 2001, synthetic siRNAs have been reported to be involved in RNAi initiation in human cell line (Elbashir et al. 2001). This discovery has been revolutionizing the field of gene-function analysis.

Thus, three phenotypically different but mechanically similar types of gene silencing were described: co-suppression or post-transcriptional gene silencing

(Jorgensen 2003), gene suppression in fungi (Cogoni et al. 1996) and RNA interference in animals (Fire et al. 1998). The naturally ongoing process of RNA interference is observed in eukaryotic cells in various forms, such as formation of microRNA (Pasquinelli 2002; Bartel 2004), methylation of promoter regions (Matzke et al. 2001; Wesley et al. 2001) and heterochromatinization (Schramke and Allshire 2003). The evolutionary functions of RNA gene silencing and the related processes are designed to protect the genome against invasion of mobile genetic elements, such as viruses and transposons, as well as for the synchronous functioning of the developmental programs in eukaryotic organisms.

23.2 Classes of RNAs That Induce Gene Silencing

Many key biological processes are explicitly dependent on RNAs, which exist as molecules with various functions. Besides the ribosomal RNA (rRNA), transfer RNAs (tRNAs) and messenger RNAs (mRNAs), which are the most abundant types of RNAs inside the cell, another four basic classes of RNAs are present. These classes are hairpin RNAs (hpRNAs), double-stranded RNAs (dsRNA), small interfering RNAs (siRNAs), and microRNAs (miRNAs) and take active roles in RNA silencing pathway (Agrawal et al. 2003).

Double-Stranded RNA (dsRNA) dsRNA is a class of RNAs formed by the complementary base pairing of two single-stranded fragments of RNA (Agrawal et al. 2003). dsRNAs are naturally found in the cell and are generally derived from the replacement of transposons (Schramke and Allshire 2003) or virus induction. dsRNA is as well produced during virus replication and acts as an initiation molecule for the RNA gene silencing pathway. dsRNAs are recognized by the plant as "non-self" (or foreign ones) and are subjected to cleaving by cell Dicer-like enzymes. As a result, small fragments of RNA are produced, which are known as small interfering RNAs (siRNAs) (Hammond et al. 2000; Zamore et al. 2000; Hutvagner et al. 2001).

Small Interfering RNA (siRNA) siRNA, also known as short interfering RNA or silencing RNA, is a class of double-stranded RNA molecules with length of 20–25 base pairs and a two nucleotide 3′ overhangs, responsible for their various functions in the cell. siRNA is involved mainly in RNA interfering pathway being its basic component (Denli and Hannon 2003; Hamilton and Baulcombe 1999). siRNA disrupts a gene function via interfering with the RNA expressed by that gene by initiating a complementary-specific degradation of mRNA or blocking of translation (Martinez et al. 2002). Small interfering RNAs are believed to have an important role in viral resistance and in preventing transposons transposition (Lippman et al. 2003).

Short Hairpin RNA (shRNA) shRNA is another type of dsRNA with a hairpin turn and a length of 21 nt, derived from a long fragment of single-stranded RNA (ssRNA) containing an inverted repeat (Wesley et al. 2001). Expression of shRNA is typically a result of a plasmid or a vector introduced in the cell. A constitutive promoter, U6 in animal cells or Cauliflower mosaic virus 35S promoter (CaMV35S) in plants, is required to ensure the continuous expression of shRNA. Hairpin RNA is transcribed by RNA polymerase III in animal cells and by RNA polymerase II in plant cells into a primary transcript of about 70 nt, which is processed by RNase III enzyme to produce the characteristic hairpin structure in the nucleus. After that, the pre-shRNA molecule is exported into the cytoplasm, where it is processed by Dicer enzyme to mature shRNA and bound to RISC to activate gene silencing (Paddison et al. 2002).

Micro RNAs (miRNAs) miRNAs are small non-coding single-stranded molecules of about 21–23 nucleotides in length which negatively regulate gene expression. miRNAs are formed from precursor single-stranded RNA transcripts with the ability to fold back to produce imperfectly double-stranded stem loop precursor structures (Grosshans and Slack 2002). miRNAs were first discovered in *C. elegans*, while screening for genes controlling developmental timing (Lee et al. 1993). Hundreds of miRNAs have been identified in plants and animals, including the hundreds unique miRNA from *Arabidopsis* alone (Lee and Ambros 2001; Llave et al. 2002; Reinhart et al. 2002; Millar and Waterhouse 2005). The main function of miRNA is gene regulation as they mimic the role of siRNAs (Grosshans and Slack 2002). miRNAs have been found out to possibly target about 60% of the genes of mammals including humans (Lewis et al. 2005; Friedman et al. 2009). miRNAs are evolutionarily conserved, probably due to their vital role in gene regulation (Axtell and Bartel 2005; Tanzer and Stadler 2004).

Piwi-Interacting RNA (piRNA) piRNA represents the largest class of small non-coding RNA molecules. They are expressed in animal cells and are derived from a large variety of sources, including repetitive DNA and transposons (Klattenhoff and Theurkauf 2008). piRNA are complex, larger in size than miRNA (26–31 nt) and lack conservation. piRNAs appear to act both at post-transcriptional and chromatin levels (Seto et al. 2007). piRNAs interact with the so called "piwi" proteins – a part of the family of the Argonautes to form RISC and direct it to their transposon targets (Aravin et al. 2008).

Repeat Associated Small Interfering RNA (rasiRNA) rasiRNA is considered to be a 24–29 nt long subtype of piRNA (Gunawardane et al. 2007; Faehnle and Joshua-Tor 2007), which targets transcripts from repeat sequences and silences transposons and retrotransposons (Dorner et al. 2007; Klattenhoff et al. 2006) and

is itself generated by annealing of sense and antisense related transposable elements (Aravin and Tuschl 2005). RasiRNA does not require Dicer and acts by a mechanism, known as ping-pong (Klattenhoff et al. 2006).

Tiny Noncoding RNAs (tncRNAs) The first tncRNAs were discovered in *C. elegans.* They are derived from noncoding sequences and are very similar to miRNAs with regard to their size, single-stranded structure and lack of a precise complementarity to a given mRNA. However, they lack the hairpin precursor and are phylogenetically non-conservative. The developmental role of tncRNA is not fully understood (Ambros et al. 2003).

23.3 Mechanism of PTGS

The main function of RNA gene silencing is cytoplasmic degradation of a specific complementary RNA molecule. DsRNA or small interfering RNA (siRNA) cannot alone degrade mRNA. Several factors associated with gene silencing are involved in the process: Dicer-like proteins, proteins of the Argonautes family and RNA helicases. An intermediate element in the metabolic pathway of RNA gene silencing is dsRNA and the degradation of this element triggers the RNA gene silencing process. A Ribonuclease III-like enzyme called Dicer recognizes and decomposes the mRNA into siRNAs. Subsequently, these mRNAs serve as leaders for the degradation of homologous RNA molecules mediated by the RNA-induced silencing complex (RISC) (Fig. 23.1).

The mechanism of RNA gene silence is a two-stage process (Fig. 23.1). The first stage involves binding of Dicers to large dsRNAs and their cleaving to 21–23 bp fragments to form siRNAs. During the second stage, these siRNAs join the multinuclease RISC complex which degrades homologous single-stranded mRNAs. The first class of RNAs, which play an active role in gene silencing, are the dsRNAs formed by the complementary base pairing of the two ssRNA fragments (Agrawal et al. 2003). The long dsRNAs are produced by the movement of mobile genetic elements (Schramke and Allshire 2004) or by viral induction which initiates the process of gene silencing (Rovere et al. 2002). dsRNAs cannot alone degrade mRNA but need two types of enzymes – the Dicers and the RISC complex. Dicers were found for the first time in *Drosophila* and are complex enzymes belonging to the RNase III family (Bernstein et al. 2001). The Dicer has four different domains with different functions: (a) N-terminal domain – helicase, (b) double RNase III, (c) C-terminal – dsRNA binding domain, (d) PAZ (Piwi/Argonaute/Zwille) domain (Kuznetsov 2003; Arenz and Schepers 2003). The PAZ domain of the Dicer physically interacts with the corresponding PAZ domain of the RISC complex. The two RNase III domains cut the dsRNA forming the characteristic 5′-phosphate and 3′-hydroxyl residues of the resulting siRNAs. AGO1 (a protein from the Argonautes family) mediates the mRNA cleavage by target micro RNAs and in the maintenance of the chromatin structure (Vaucheret 2006), a mechanism which is ATP

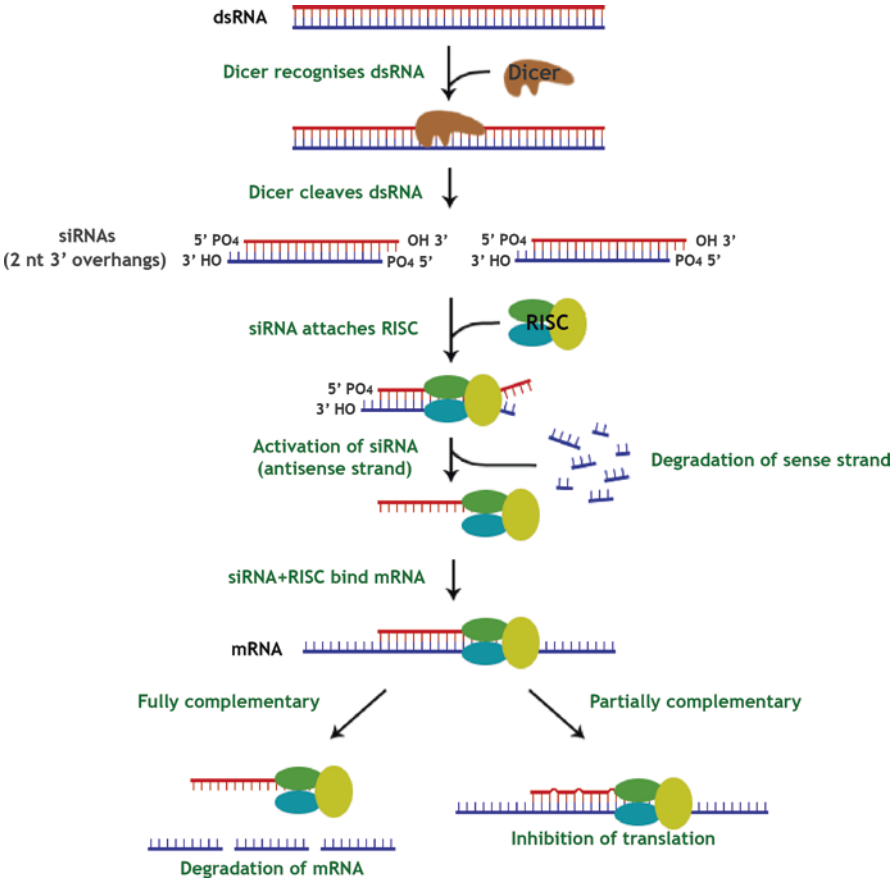

Fig. 23.1 Mechanism of post-trascriptional gene silencing (RNAi)

independent (Kuznetsov 2003). The helical domain is also involved in the process. The Dicer proteins function in two different ways in silencing a gene by recognizing different types of precursor dsRNAs. The first mechanism includes cleavage of dsRNAs originating mainly from the protein-encoding gene and thus, generates double-stranded siRNAs which direct the subsequent endonuclease cleavage of homologous RNAs during a fully complementary interaction (Elbashir et al. 2001). In the second mechanism, the Dicer cuts the not fully complementary RNA duplexes, derived predominantly from the regions between the protein-encoded genes, into short RNAs which subsequently convert into micro ribonuclease complexes (miRNPs) to further regulate inhibition of translation or other effects associated with the gene silencing (Hutvagner et al. 2001; Voinnet 2002). Accordingly, the Dicer processes the precursors – dsRNAs into siRNAs and microRNAs. There are four types of the enzyme involved in the biogenesis of siRNAs – DCL1 (microRNAs), DCL2 (viral

RNAs), DCL-3 (endogenous siRNAs), and DCL-4 (exogenous siRNAs) (Xie et al. 2004, 2005), which leads to production of different size of siRNAs.

The RISC complex is a component of the gene silencing apparatus which uses siRNAs to trace and degrade target mRNAs. The RISC complex was first detected in *Drosophila* and consisted of protein and RNA (Hammond et al. 2000). The protein component of the complex has ribonuclease activity capable of decomposing RNA. In addition to ribonucleic activity, RISC also contains a PAZ domain involved in regulating RNA gene silencing. Additional components of the RISC complex are two RNA binding proteins – intron and dFMR protein (Arenz and Schepers 2003) and the DCR-2/R2D2 complex, which also facilitates the inclusion of siRNAs in the RISC complex (Liu et al. 2006). RISC uses siRNAs in search of a complementary target sequence in mRNA. The sequence and structure of the siRNA determines which of its two chains will participate in the mechanism of gene silencing. Each siRNA dissociates from the active center of the Dicer and one of its chains selectively enters the RISC complex, while the other degrades. The degradation process is initiated after the successful location and cleavage of the complementary mRNA region by the siRNA-RISC complex (Fig. 23.1).

The siRNAs lead the RISC complex to bind to the target mRNA and suppress its translation more than its degradation (Fig. 23.1). Translational suppression occurs at a certain stage after translation initiation, because the ribosomes along the suppressed mRNA are undergoing active translation (Vaucheret 2006). These RNAs are of two different groups of 21–22 bp and of 25 bp (Elbashir et al. 2001; Aigner 2006) with a 5′-phosphate and a 3′-hydroxyl residue attached to a 2-base loop (Lipardi et al. 2001). The 3′-hydroxyl group is needed for targeting gene silencing in vitro (Lipardi et al. 2001). However, it is unlikely that both groups of siRNAs have the same function, since they accumulate in different way in locally and systemically genetically silenced tissue (Lipardi et al. 2001). siRNAs of 21–22 bp direct the RISC ribonuclease to the target of RNA gene silencing (Elbashir et al. 2001; Aigner 2006). The pathways of the gene silencing are divided into those that require RNA-dependent RNA polymerase (RdRPs) and those that do not depend. The presence of RNA-dependent RNA polymerase (RdRp) is required for RNA gene silencing in *C. elegans* and *Neurospora crassa* (Sijen and Kooter 2000).

Two models were proposed to explain the role of RdRPs. One explains the role of RdRPs by using primary siRNAs to induce synthesis of dsRNA using the target mRNA as a template. Propagation of siRNAs is associated with systemic gene silencing, a phenomenon observed in worms and plants (Boerjan et al. 1994; Fire et al. 1998). The mechanism of *in-trans* gene silencing is based on the presence of siRNAs which correspond to the homologous region between the gene silencing inducer and the target RNA (Depicker and Montagu 2003).

Several studies in plants have shown that gene silencing may spread to regions downstream from the 5′ to 3′ of the target RNA chain (Braunstein et al. 2002; Vaistij et al. 2002). A remarkable feature of gene silencing is that it is not a cellular autonomous process. The process can be induced in a tissue that actively expresses a transgene by a mobile transmission signal originating from a tissue where the same transgene is silenced (Jorgensen et al. 1998; Fagard and Vaucheret 2000). The signal

is sequence-specific and moves one-way (Voinnet and Baulcombe 1997; Sonoda and Nishiguchi 2000). The propagation of gene silencing signal depends on the transcription of target RNA and RdRp SDE1/SGS2 (Baulcombe 2002).

23.3.1 Dicers

Naturally occurring small RNAs may be: (1) endogenous siRNAs; (2) microRNAs involved in gene regulation; (3) siRNAs derived from transposons; (4) siRNAs derived from viruses. All siRNAs are products of the degradation of long dsRNAs and of RNAse III-like enzymes, discovered for the first time in *Drosophila* (Bernstein et al. 2001). These enzymes are called Dicer in animals or Dicer-like (DCL) in plants. They are multifunctional proteins and contain one or more dsRNA binding domains. The number of Dicers may vary in different organisms. Many animals encode only one Dicer (also humans), *Drosophila* encodes two Dicers (Lee et al. 2004), *Arabidopsis thaliana* – four DCL homologues (DCL1, DCL2, DCL3, and DCL4) (Schauer et al. 2002). DCL1 is primarily responsible for the processing of microRNAs (Herr et al. 2005). Some other factors, such as HEN1 and HYL1 (dsRNA-binding protein), also help DCL1 in the generation of microRNAs (Vazquez et al. 2004; Xie et al. 2004). HEN1 is involved in some other functions such as natural viral resistance and transgenic silencing (Boutet et al. 2003). DCL2 is involved in the production of 22-nucleotide viral siRNAs (Gasciolli et al. 2005; Chen et al. 2018), systemic spread of gene silencing (Chen et al. 2018) and in antiviral protection (Gasciolli et al. 2005). DCL2 is required in the target tissue to respond to the PTGS signal (Chen et al. 2018). The production of viral siRNA requires two RNA-dependent RNA polymerases (RDR1 and RDR6) depending on the virus type (Muangsan et al. 2004; Xie et al. 2004). DCL3 together with RDR2 play a role in the generation of endogenous siRNAs (24 bp) and RNA-dependent DNA methylation (Xie et al. 2004). These endogenous siRNAs are involved in the initiation or maintenance of the state of the heterochromatin (Matzke et al. 2004). DCL4 participates in the production of 21-nucleotide siRNAs as a signal for the propagation of cell-to-cell gene silencing (Dunoyer et al. 2005). DCLs proteins have interchangeable and overlapping functions in both pathways of siRNAs and microRNAs (Deleris et al. 2006) and act in a coordinated DCL genetic pathway (Chen et al. 2018).

23.3.2 RNA-Induced Silencing Complex (RISC)

The RISC complex is a multi-protein complex which participates in various catalytic functions in the RNA gene silencing process such as mRNA degradation and translation inhibition. Specific sequence recognition of mRNA is performed by siRNAs. Another protein called Argonate (AGO) is part of the RISC complex and occurs in all organisms. AGO protein is essential for the nuclease activity of the complex. Ten members of the Argonauts family were identified. The AGO1 protein

is regulated by the microRNA (miR168), indicating that it regulates its own expression by negative feedback loop (Vaucheret et al. 2004). AGO1 participates in the cleavage and processing of microRNAs and some classes of endogenous siRNAs (Baumberger and Baulcombe 2005). The role of AGO4 is in the production of long siRNAs of about 24 nucleotides and mediating the change of chromatin by methylation of histones (Zilberman et al. 2003). AGO2 is part of the RISC complex and is essential for the siRNA-directed RNA gene silencing. AGO2 plays no role in the processing of microRNAs (Okamura et al. 2004). Most AGO proteins, if not all, which are involved in the different stages of RNA gene silencing, also determine the mode of action of the RISC complex in which they are integrated (Baulcombe 2004).

23.3.3 Spread of RNAi

RNAi signals in plants can transmit from cell to cell, allowing systemic "immune" response. Mechanisms and their regulation in plants are still unclear. However, recent studies put some light onto some participants in this process. An enzyme of the Dicer-like group (DCL2) was found to be essential in the target tissue to respond to the PTGS signal (Chen et al. 2018). Only recently, two receptor-like kinases (RLK) 'Barely Any Meristem' 1 and 2 (BAM1 and BAM2) in the plasma membrane and plasmodesmata were established to play a redundant role in cell-to-cell signalling of RNAi. The C4 protein of Tomato yellow leaf curl virus can interact with these kinases, which results in inhibition of the intercellular spread of RNAi. However, whether BAM1/BAM2 can bind siRNA-binding proteins (AGOs) or siRNA molecules directly is not yet determined (Rosas-Diaz et al. 2018).

23.4 Application of Gene Silencing in Plants

RNA silencing is an adaptive immune response that restricts accumulation or spread of viruses (Morris and Rossi 2006). To counteract or evade host defenses mediated by RNA silencing, viruses have evolved numerous strategies such as the deployment of decoy RNAs, specialized replication mechanisms, and sequestration of viral RNAs in large protein or membrane complexes (Ghoshal and Sanfacon 2015; Nie and Molen 2015; Liu et al. 2017). Almost all plant viruses encode viral suppressors of RNA silencing (VSRs), which interfere with host RNA silencing in addition to their functions in viral replication, encapsidation, or movement (Burgyan and Havelda 2011; Wang et al. 2012; Liu et al. 2017), thus contributing to viral symptoms. VSR-mediated inhibition of RNA silencing generally occurs in two ways: indirectly, by binding to short or long dsRNAs and thus, sequestering small RNA duplexes, which results in the suppression of the assembly of AGOs into RISCs, and directly, by interaction with AGO1, which prevents siRNA or miRNA loading, impedes slicing activity, or degrades the AGO1 protein (Silhavy and Burgyan 2004;

Burgyan and Havelda 2011; Wang et al. 2012; Moon and Park 2016; Liu et al. 2017).

Uses of PTGS until the end of 1980s, which included only modifications of DNA or protein leading to transcriptional repression or activation, were classified as epigenetic drugs. During the 1990s, the discovery of a number of gene-silencing phenomena at post-transcriptional level in plants, fungi, and animals, introduced the concept of PTGS or RNA silencing (Baulcombe 2000; Matzke et al. 2001). The application of dsRNA technology for large-scale investigation of gene functions can be made possible by enhancing the critical experimental procedures in terms of speed and efficiency (Wesley et al. 2001; Brummell et al. 2003).

Knowledge of PTGS was mainly used for construction of transgenic plants (Eamens et al. 2008). In 2014, Sahu et al. identified single-stranded DNA satellite associated with Croton yellow vein mosaic begomovirus (CYVMV) in croton plants which carries genes explicit for the packaging, replication, insect transmission and movement in plants. The researchers used both transgenic systems and transient *Agrobacterium tumefaciens*-based delivery in plants to induce gene silencing against the virus. Plants, carrying an intron-hairpin construct covering the gene βC1, accumulated cognate small-interfering RNAs and remained symptom-free after exposure to CYVMV and its satellite (Sahu et al. 2014). Zhang et al. (2018) used the CRISPR-Cas9 system from the bacterium *Francisella novicida* to genetically modify *Nicotiana benthamiana* and *Arabidopsis* plants. The transgenic plants expressed RNAs specific for the cucumber mosaic virus (CMV) or tobacco mosaic virus (TMV), which resulted in attenuated virus infection symptoms and reduced viral RNA accumulation. RNAi technology was also used for control of Tomato yellow leaf curl virus via silencing a part of its genome and thus, reducing loss of plant production (Dhakar et al. 2010).

However, PTGS can be utilized as a tool for control of viruses in plants by targeting a specific conservative region from the virus genome for silencing without invading the plant genome. Epigenetic control of viruses in plants by using artificial RNAs is a promising novel alternative strategy. PTGS was induced in tobacco plants (Petrov and Stoyanova 2011; Petrov 2012) and potato plants cv. Agria by specific siRNAs for HC-Pro region of Potato virus Y (PVY) strain NTN to effectively block the viral replication (Petrov et al. 2015a). The selected region was amplified with previously designed primers with promoter sequences linked to both sides of the target sequence using high-fidelity PCR. The PCR primers were designed so that they contained RNA polymerase promoter sequences at their 5′-ends. Thus, in the PCR product, RNA polymerase promoter sequences flanked the target sequence. In addition, each primer should contain 17–22 nucleotides of the gene-specific target sequence at the 3′-end. dsRNA was synthesized by the combination of in vitro transcription and replication of DNA template according to the instructions of Replicator RNAi Kit, Finnzymes, Finland (Petrov and Stoyanova 2011; Petrov 2012; Petrov et al. 2015a, b) (Fig. 23.2).

PTGS was induced in potato plants cv. Arinda also by specific dsRNAs and siRNAs for HC-Pro region of PVY, which effectively reduced systemic spread of the

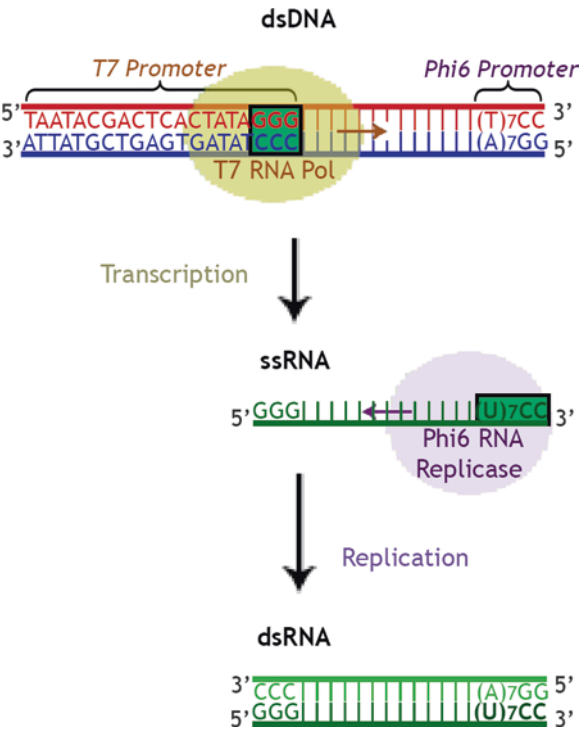

Fig. 23.2 Production of dsRNAs in vitro

virus. Reduction of the expression of the HC-Pro gene of PVY^N in newly grown leaves of potato plants, and hence, the viral replication in all inoculated plants with the virus was established. The old leaves of the potato plants treated and inoculated with PVY remain infected and later defoliate. All newly formed leaves of potato plants cv. Arinda grown after treatment with dsRNAs and siRNAs, and subsequent PVY inoculation remain virus-free (Petrov et al. 2015b).

The investigations with treatments with virus-specific siRNAs resulted in effective control of the replication and the systemic spread of the virus (Petrov and Stoyanova 2011; Petrov 2012; Petrov et al. 2015a, b). The used strategy allows direct control of the viral infection in plant protection, representing unique opportunities without alternatives. RNA treatments can be adapted towards the specific pathogen in target, regardless of the specific crop or variety. The acquired resistance is not inherited, which allows the preservation of sensitive crop varieties with valuable nutrition and taste properties. The production remains ecologically clean from chemical compounds, transgenic proteins, allergens, mutagens, etc., which may pose treats to human health.

23.5 Conclusions

Small interfering RNAs efficiently inhibit gene expression in eukaryotic cells. Introduced into cells they elicit induction of Post-transcriptional gene silencing (PTGS) known as RNA interference. They are incorporated into a RNA-induced silencing complex (RISC). The antisense strand guides RISC to the complementary target pathogenic mRNA and induces its endonucleolytic cleavage. PTGS is a mechanism of gene regulation in eukaryotic organisms. It is similar to humoral immunity, which protects eukaryotes against viruses and transposons. PTGS can be utilized for controlling gene expression of pathogenic genes in order to stop pathogen invasion in the host plant. Treatments with siRNA feature some unique characteristics: specific pathogen targeting, ability to spread throughout the plant, and lack of genome modifications, which allow safe releasing of the plants from disease.

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Plant Platform for Therapeutic Monoclonal Antibody Production

24

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Abstract

Plant cells have protein synthesis and post-translational modification (glycosylation and phosphorylation) mechanisms similar to those of animal cells therefore the development of biotechnology allows considering plants as factories for therapeutic proteins, including therapeutic monoclonal antibodies (TMAs). The plant monoclonal antibody production platform has attracted researchers' attention due to its flexibility, speed, scalability, low production costs, and absence of a risk of contamination by pathogens of animal origin. Modern methods for the production of therapeutic proteins are based on stably transformed transgenic plants and the transient expression of foreign genes. This chapter considers modern methods for obtaining TMAs produced in plants (P-TMA), features of the carbohydrate composition and methods for humanising the carbohydrate profile of P-TMAs. Examples of P-TMAs that have successfully passed preclinical trials and have a perspective in clinical use are given. The prospects of P-TMAs are determined by the economic benefits and speed of production, which are especially important for individualised cancer therapy, as well as cases of bioterrorism and pandemics.

Keywords

Monoclonal antibody · Immunoglobulin G · Antibody N-glycosylation · Therapeutic antibody · Immunotherapy · Plant viruses · Transient expression

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24.1 Introduction

Plants are an ancient source of medicines for humans, but only after the emergence of biotechnology have they been seriously considered as factories for therapeutic proteins. The term "molecular plant farming" refers to the production of therapeutic proteins in plants and is based on the similarity of plant and animal cell protein expression and post-translational modification mechanisms including glycosylation and phosphorylation (Fischer et al. 2012; Habibi et al. 2017; Loh et al. 2017; Moustafa et al. 2016; Sack et al. 2015; Stoger et al. 2014; Tschofen et al. 2016). Molecular pharming has a number of advantages compared with bacteria, yeast and animal platforms including the (a) production of low-cost biomass; (b) the absence of contaminants that are toxic or infectious to humans in the final products; (c) the accumulation of complex proteins with proper folding; and (d) using simple protein purification methods.

Until recently, the plant-based pharmaceutical industry was still at an early stage of development. An important milestone was the approval by the US Food and Drug Administration in 2012 of the plant-made taliglucerase alfa (ELELYSO), which Protalix BioTherapeutics produced in a suspension culture from a transgenic carrot cell line for the treatment of patients with Gaucher disease (Grabowski 2008). Twenty years ago, plants were considered as an alternative to animal cells and as a factory for low-cost and virus-free recombinant medicinal preparations obtained in the culture of mammalian cells (Stoger et al. 2014). Now, biotechnologists are attracted by the possibility of the production of human blood proteins (e.g., human serum albumin) and antibodies in plants, including plant-made therapeutic monoclonal antibodies (P-TMAs) against cancer and human immunodeficiency virus. The advantages of plants are superior to those of animal cells in the production of (a) P-TMA, the glycosylated forms of which have enhanced efficacy (Jennewein and Alter 2017; Lalonde and Durocher 2017; Loos and Steinkellner 2014); (b) vaccine preparations that are produced rapidly during, for example, an epidemic of influenza or for the treatment of non-Hodgkin lymphoma (Bendandi et al. 2010); (c) an edible vaccine (Concha et al. 2017) or microbicides (Barbieri et al. 2017); (d) human proteins obtained in multitone amounts (human serum albumin) (He et al. 2011) or, conversely, for a small group of patients, for example, those suffering from Gaucher disease (Naphatsamon et al. 2018). Plant production platforms include (a) suspension plant culture (Holland and Buyel 2018; Sukenik et al. 2018); (b) transgenic plants obtained through stable plant cell transformation (Tschofen et al. 2016), which includes both whole plants and root culture (Zhou et al. 2011), and (c) transient expression (Komarova et al. 2010). The latter platform is an alternative to stably transformed plants and allows obtaining a number of important pharmaceutical proteins very quickly, and, most importantly, for individualised cancer therapy, as well as in cases of bioterrorism and pandemics.

In this chapter, we will consider in detail the plant platform for the production of P-TMAs, as well as plant biosimilars to TMAs directed against infectious and cancerous diseases.

24.2 Brief Description of Human Antibodies

There are five classes of immunoglobulins (IgM, IgG, IgE, IgD and IgA) in the human body, among which there are two subclasses for IgA and four subclasses for IgG (Altshuler et al. 2010; Vidarsson et al. 2014) (Table 24.1). After secondary immunisation, B-lymphocytes predominantly produce IgG molecules, which unlike other immunoglobulins can penetrate into the placenta, and similar to IgA, be secreted with human milk. IgG molecules consist of two 50-kDa heavy γ chains and two 25-kDa light chains belonging to either of the two types, kappa (κ) and lambda (λ) (Table 24.1). The IgG class is divided into four subclasses by their relative concentration in normal human serum, where IgG1, IgG2, IgG3 and IgG4 are approximately 66%, 23%, 7% and 4% respectively of the total serum IgG (Nezlin 1998).

The subclass of human IgG1 is preferred for development of therapeutic antibodies because this type of immunoglobulin has a long half-life in the blood (Table 24.1) and exhibits more pronounced effector properties than the other classes and subclasses of human immunoglobulins (Irani et al. 2015; Vidarsson et al. 2014). Compared with other antibodies, IgG is relatively simple and is a homodimer having a molecular weight of 150 kDa and consisting of a monomer with two polypeptide chains, heavy and light, linked together by an interchain disulfide bond. In full-length immunoglobulin G, these two monomers are combined due to the formation of disulfide bonds between the heavy chains (Fig. 24.1). The following three structural parts are distinguished in IgG (Altshuler et al. 2010; Irani et al. 2015; Vidarsson et al. 2014): (a) a Fab fragment ("fragment, antigen binding" or "antigen binding fragment") of an antibody consisting of variable domains from two light (V_L) and heavy chains (V_H) forming paratopes, as well as the two nearest constant domains form light and heavy chains (C_H1 and C_L1); (b) the C-terminal structure, referred to as the Fc fragment (fragment crystallisable), which includes the heavy chain constant domains C_H2 and C_H3; and (c) the hinge region (HR), whose flexible structure provides the mobility of antibody fragments with respect to one another (Fig. 24.1). The length and flexibility of the HR varies widely among IgG subclasses, which affects the conformation of Fab with respect to the Fc domain. The IgG1 HR is 15 amino acids and is very flexible. In this region, the IgG heavy chains are covalently linked by disulfide bonds (2 for IgG1 and IgG4, 4 for IgG2 and 11 for

Table 24.1 Human immunoglobulins

	IgG							
	IgG1	IgG2	IgG3	IgG4	IgA	IgM	IgD	IgE
Molecular formula	$\kappa_2\gamma_2$	$\kappa_2\gamma_2$	$\kappa_2\gamma_2$	$\kappa_2\alpha_2$	$\kappa_2\alpha_2$	$\kappa_2\mu_2$	$\kappa_2\delta_2$	$\kappa_2\varepsilon_2$
	$\lambda_2\gamma_2$	$\lambda_2\gamma_2$	$\lambda_2\gamma_2$	$\lambda_2\alpha_2$	$\lambda_2\alpha_2$	$\lambda_2\mu_2$	$\lambda_2\delta_2$	$\lambda_2\varepsilon_2$
Subclasses	γ1	γ2	γ3	γ4	α1-2	–	–	
Molecular weight (kDa)	146	146	170	146	385	950	185	190
Carbohydrate content (%)	3	3	3	3	7.5	12	12	12
Half-life (days)	21	21	7–21	21	5–6	5	2,8	2,5
Transplacental transport	++++	++	++/+++	+++	–	–	–	–

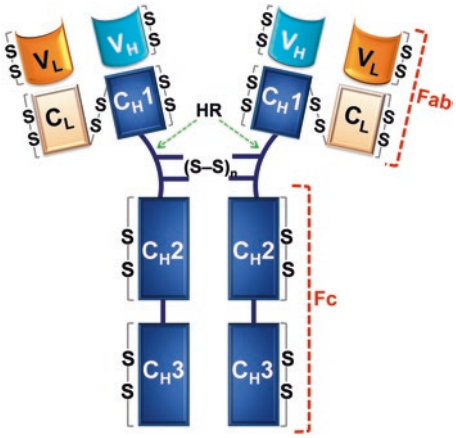

Fig. 24.1 Main structural elements of IgG. The antigen-binding fragment (Fab) connects to the Fc-domain via a hinge region (HR). V_L and C_L are the variable and constant domains of the IgG light chain, respectively. V_H and $C_{H1–3}$ are the variable and constant domains of the IgG heavy chain, respectively. S-S designates intra- and intermolecular disulfide bonds that stabilise the antibody

IgG3), while the C_H2 and C_H3 domains contain noncovalent bonds between the chains. Depending on the isotype, dimerisation of the two halves of the antibody involves the formation of 2–11 disulfide bonds between the heavy chains, which stabilise the structure of IgG due to intra- and intermolecular "piercing" of the heavy and light chains. Two intracellular disulfide bonds stabilise each light chain and four for each heavy chain. All these interactions provide stability and contribute to the length of the antibody half-life. Another factor that determines the relatively long half-life of an antibody in the human body (Table 24.1) is the ability of the antibody Fc region to bind to specific receptors (Chan et al. 2015; Nimmerjahn and Ravetch 2008). The antibody Fc region defines such antibody effector functions as antibody-dependent cellular cytotoxicity (ADCC) (Román et al. 2013), complement-dependent cytotoxicity (CDC) (Lindorfer et al. 2014), antibody-dependent cellular phagocytosis (ADCP) (Gül and van Egmond 2015) and anti-inflammatory activity (Nimmerjahn and Ravetch 2008).

Like most glycoproteins, healthy human immunoglobulins undergo glycosylation in the endoplasmic reticulum (ER) and Golgi apparatus (Vidarsson et al. 2014). In different classes of immunoglobulins, the degree of glycosylation varies; for IgG, the glycans constitute approximately 3% of the antibody mass, which is relatively low compared to IgM, IgD and IgE (Table 24.1). In all classes of immunoglobulins, N-linked glycans are detected in both the Fc fragment and the Fab. O-glycans are found in the HR of IgD, IgA and IgG3 (Plomp et al. 2015). Polyclonal IgG from human serum mainly contains complex type N-glycans. The Fc and Fab fragment glycans differ considerably in the content of specific residues, although they have a common biantennary structure, i.e., consist of a branching heptasaccharide and two disaccharide blocks (antennas). The presence of large multi-antenna glycans, such as tri- and tetra-antenna glycans, in the healthy human IgG was not detected.

24.3 TMA Production in Animal Cells

The method of converting B-lymphocytes into immortal cells developed by Köhler and Milstein (1975) formed the basis for the technology for the production of monoclonal antibodies in cell culture. Mouse monoclonal antibodies, such as ortoclone OKT3 (Muromonab, anti-CD3), which prevents the rejection of foreign organs in kidney transplantation, was the first TMA (Smith 1996). During the next stages of the development of this technology, the problems of hybridoma instability were solved and the techniques for "humanising" mouse antibodies were created, which removed the problem of induction of anti-mouse antibodies in patients (Cymer et al. 2018; Kunert and Reinhart 2016). Although the alternative to the hybridoma approach has been the biosynthesis of antibodies or their fragments by genetic engineering or using heterologous expression systems in bacteria, yeast, insect and mammalian cells, TMAs are currently mainly produced in animal cells, as these cells provide for the synthesis of antibodies close to the human carbohydrate composition. TMAs are produced in one of the three following cell lines (Cymer et al. 2018; Dorokhov et al. 2016; Komarova et al. 2017a): Chinese hamster ovary (CHO) tumour cells, mouse NS0 (non-secretory murine myeloma cells) or SP2/0 (mouse myeloma cells), and less often in the HEK293 and PER.C6 human cell lines. The CHO cell line is the main "workhorse" for the production of more than 70% of therapeutic proteins. The reason is that CHO is immune to human viruses and provides the highest level of antibody production, reaching a titre of 10 g/l in a fed-batch system (medium-enriched process) (Alsayyari et al. 2018; Zboray et al. 2015). CHO cells can be genetically modified and are now the most popular for the post-translational modification of TMAs, namely, their glycomodification, including fucosylation and sialylation (Cymer et al. 2018; Dorokhov et al. 2016; Komarova et al. 2017a).

The NS0 and SP2/0 mouse lines provide a tenfold lower yield of antibodies compared to CHO cells (Ho et al. 2012). Human HEK293 cells are more often used for the transient expression of genes encoding antibody chains, and their antibody yields are two to three times lower compared to CHO cells. It is believed that the achieved antibody yield parameters in the animal cell system (0.5–8.0 g/l) may eventually be surpassed as a result of (a) improvement in vector systems (Kunert and Casanova 2013), (b) gene modification of cells aimed at enhancing cellular biosynthetic activity by decreasing the probability of apoptosis, (c) increasing the translation efficiency of target gene mRNAs (Kunert and Reinhart 2016), and (d) improving the secretion of antibodies by cells (Le Fourn et al. 2014).

In addition to the relative simplicity of cultivation, mammalian cell lines are also used for TMA production because the antibodies produced therein are characterised by a carbohydrate profile that is closest to human serum IgG (Butler and Spearman 2014; Hossler 2012). An antibody synthesised in an animal cell contains more than ten monosaccharides, among which the Asn297-linked glycan is the most studied (Cymer et al. 2018; Dorokhov et al. 2016; Komarova et al. 2017a). However, the heterogeneity of carbohydrate composition in TMA preparations obtained even in the same culture was revealed. For example, Rituximab is highly heterogeneous in

the glycosylation of the Fc fragment (Pierpont et al. 2018; Schiestl et al. 2011). In addition to the specificity of metabolism of an individual cell, the heterogeneity of TMAs is also contributed by the elimination of carbohydrate residues by extracellular glycosidases released by the destroyed producer cells, which is especially characteristic for batch and fed-batch production systems (Blondeel and Aucoin 2018). In contrast, the high productivity of the cell line contributes to the appearance of immature high-mannose forms of antibodies that are formed in the early stage of glycosylation prior to the addition of N-acetylglucosamine (GlcNAc) or fucose. It is suggested that with high antibody production in the Golgi apparatus of the cell line, deficiency of the system components may occur due to the insufficient rate of substrate transfer through cellular and intracellular membrane barriers or the insufficient activity of glycosylation enzymes (Zalai et al. 2016). High-mannose forms of antibodies have an increased rate of clearance of the drug from the body (Reusch and Tejada 2015; Sha et al. 2016).

The disadvantage of TMA production systems in mammalian cells other than human is the synthesis of immunogenic glycans, which cause undesirable immunological reactions (Cymer et al. 2018; Dorokhov et al. 2016; Komarova et al. 2017a). Among them, is the presence of galactose-α1,3-galactose (α-Gal) and N-glycolylneuraminic acid (Neu5Gc) located in the terminal portion of the glycan. For example, administration of Cetuximab from SP2/0-derived mouse cells containing α-Gal and Neu5Gc epitopes to patients can sometimes cause severe anaphylactic shock accompanied by the induction of IgE specific for α-Gal (Chung et al. 2008). It should be noted that even in the body of a healthy person, IgE to the α-Gal and Neu5Gc epitopes are present (Padler-Karavani and Varki 2011). Although CHO cells contain enzymes necessary for the formation of these immunogenic epitopes (Bosques et al. 2010), there is currently no documented evidence of adverse immunological effects due to the administration of TMAs synthesised in CHO cells to patients.

In addition to immunogenic glycans, there are other differences in the glycosylation of TMAs in producer cells (Blondeel and Aucoin 2018; Kunert and Reinhart 2016). Thus, CHO cells are characterised by a higher level of glycoprotein sialylation (Cymer et al. 2018). Increased terminal sialylation of the TMA Fc fragment, although it lowers the ADCC effector function of TMA (Scallon et al. 2007), may also have beneficial effects in patients due to anti-inflammatory effects and increased resistance of TMAs to proteolysis in the patient's bloodstream (Anthony et al. 2008; Mimura et al. 2016). Another feature of TMAs obtained in CHO culture is fucosylation (Raju 2008) and galactosylation (Blondeel and Aucoin 2018).

24.4 P-TMA Production Systems

It is difficult to create a template for the synthesis of antibodies against a specific antigen in plant cell like it happens in the early stages of differentiation of lymphocytes as a result of somatic DNA recombination (Schroeder and Cavacini 2010). It is much easier to introduce a ready-made template to ensure the synthesis of

antibodies in a plant cell. Usually, two matrices are introduced, one for the heavy chain and the other for the light chain of the antibody (Buyel 2018; Buyel et al. 2017; Sheshukova et al. 2016). Genes encoding antibody chains can be expressed in plant cells by stable nuclear genomic integration (De Neve et al. 1999), chloroplast transformation (Almaraz-Delgado et al. 2014) or by transient expression (Komarova et al. 2010, 2017a; Sheshukova et al. 2016). To obtain stably transformed plants, two methods of introducing genes encoding the light and heavy chains have been developed (De Muynck et al. 2010). The first method is based on the independent production of individual plants for the expression of light and heavy chain genes, followed by their crossing and selection of plants synthesizing both chains simultaneously (Hiatt et al. 1989). The second method consists of sequential or simultaneous introduction of genetic constructs encoding both chains (De Wilde 1996).

The first full-length monoclonal antibodies were produced with the help of the first approach (Hiatt et al. 1989), when cDNA molecules derived from mouse hybridoma mRNA were used to transform segments of a tobacco leaf, followed by regeneration of mature tobacco plants. Then, plants expressing single γ- or κ-chains of IgG were crossed and a progeny was obtained in which both chains were simultaneously synthesised. The content of the functional antibody was up to 1.3% of the total protein in the leaves. The second approach, which simultaneously introduced genetic constructs encoding both chains, was used to obtain the MAK33 antibody in the *Arabidopsis thaliana* plant (De Wilde 1996). The process of creating a fertile transgenic plant takes a considerable period of time (~1 year), but it has the advantage that cross-fertilisation can produce offspring with the desired combination of immunoglobulin chains, for example, IgA (Ma et al. 1995; De Muynck et al. 2010).

In addition to the leaves, the seeds of transgenic plants are also an attractive object for the accumulation of antibodies, where they remain stable and functional for several years even when stored at room temperature (Stoger et al. 2014). *Oryza sativa* expressing an antibody against HIV, P2G12 (the first letter designates plant), in the endosperm of a grain is an example of successful implementation of the transgenic approach (Vamvaka et al. 2016). In this work, rice zygotes were bombarded with gold particles coated with DNA encoding the heavy and light chains of the 2G12 antibody under the control of the *glutelin-1* transcriptional promoter, and plants synthesizing full-length P2G12 antibodies were selected.

Another strategy for producing antibodies is transformation of the chloroplast genome and production of transplastic plants, mainly microalgae (Bock 2015; Yusibov et al. 2016). The unicellular eukaryotic alga *Chlamydomonas reinhardtii* has attracted researchers with its safety of cultivation and the ability for controlled growth in closed photobioreactors. With the help of chloroplast transformation, it is possible to generate stable microalgae lines for the production of antibodies in a short period of time (Rasala and Mayfield 2015). However, when choosing this technology, one should consider the peculiarities of post-translational modifications of antibodies in chloroplasts.

The third strategy, the most popular to date, is based on the transient expression of genes without their integration in the genome of the host plant (Gleba et al. 2014; Komarova et al. 2010; Sheshukova et al. 2016). In the general case, when a foreign

DNA, for example, a binary vector (see below), enters the plant cell, only a small part of it will be integrated into the host chromosomes. The remaining molecules, such as episomal DNA, can remain transcriptionally active for several days. This short-term expression does not depend on chromosomal integration and position effects. For the temporal expression of antibodies, the Australian tobacco *Nicotiana benthamiana* (Goodin et al. 2008) is predominantly used.

There are several different approaches for transient expression of antibodies, but the most effective ones are based on agroinfiltration, in which a suspension of *Agrobacterium tumefaciens* is applied to the surface of a leaf in the presence of a surfactant or injected into the intercellular space of the leaf using a syringe or vacuum (Gleba et al. 2014; Komarova et al. 2010; Sheshukova et al. 2016). Agrobacterial gene transfer, including genes encoding the light and heavy chains of the antibody, from bacteria to plant cells occurs through specifically engineered vectors. This so-called binary vector functions in both *Escherichia coli* and *A. tumefaciens* cells. For example, the vector based on Bin 19 (Bevan 1984) consists of the following two parts: (a) a segment of agrobacterial T-DNA and multiple cloning sites, a marker gene for transformed plant cells, and a gene of interest (for example, genes encoding chains of the antibody) under the control of a suitable plant cell promoter; and (b) the remaining vector DNA providing the plasmid replication functions in two bacterial systems, and selectable marker genes for expression in bacteria.

Synthesis of antibody chains in a plant cell can be achieved using non-replicating vectors under the control of a promoter, which is recognised by a plant cell, such as the 35S cauliflower mosaic virus (CaMV) promoter (Lam 1994) (Fig. 24.2a). However, vectors based on the genomes of plant RNA viruses, especially the tobacco mosaic virus (TMV) and the potato virus X (PVX), were shown to be more potent and to provide higher levels of target gene expression (Gleba et al. 2014; Komarova et al. 2010; Sheshukova et al. 2016) (Fig. 24.2b). Unlike stably transformed plants that yield 1–40 μg of recombinant protein per gram of fresh leaf mass, a transient expression system based on viral vectors reaches 300–500 μg of target protein per gram of fresh leaf mass (Giritch et al. 2006).

It is important to note that for the synthesis of a multisubunit protein, the ratio in which its components are synthesised in the plant is important. In the case of an antibody, an equimolar ratio between the light and heavy chain molecules is necessary. The success of simultaneous synthesis of two chains in the same cell is determined by the elimination of template competition for cellular resources (Giritch et al. 2006; Komarova et al. 2010). RNA molecules produced from non-replicating vectors do not compete with each other for translation as the components of translation machinery (ribosomes, translation factors, etc.) are in excess in the cell. The yield of antibodies in a plant agroinjected with non-replicating vectors based on the CaMV 35S promoter depends on the stability of the mRNA in the cell. Inhibition of silencing increases the yield of antibodies several times (Garabagi et al. 2012). Thus, the presence of the silencing suppressor P19 from the tomato bushy tomato virus resulted in a yield of a trastuzumab plant biosimilar (TPB) comparable to a viral vector-based production system (Garabagi et al. 2012; Komarova et al. 2011).

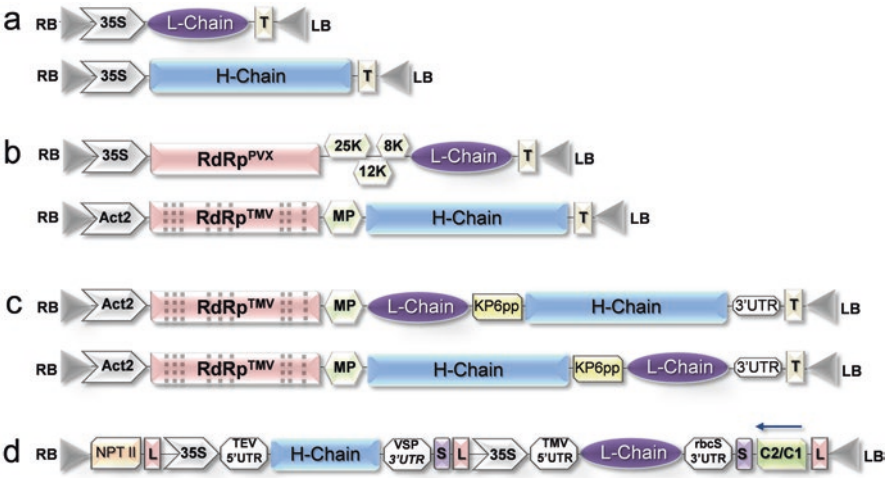

Fig. 24.2 Expression vectors used for IgG production in plants. (**a**) Schematic representation of the constructs encoding antibody heavy (H-) or light (L-) chains under control of the 35S promoter from cauliflower mosaic virus (CaMV). Individual constructs are used for the expression of the heavy and light chain genes. (**b**) Schematic representation of the viral vectors based on the tobacco mosaic virus (TMV) and potato virus X (PVX) genomes. $RdRp^{PVX}$, PVX replicase gene; 25K, 12K, and 8K genes encode a PVX triple gene block; Act2, *actin 2* gene promoter from *Arabidopsis thaliana*; $RdRp^{TMV}$, TMV replicase gene (dotted lines denote introns); and MP, TMV movement protein gene. (**c**) Two vectors that control the synthesis of the heavy and light chain genes of the VRC01 monoclonal antibody are schematically depicted. KP6pp, a sequence encoding a recognition site for the kex2p-like protease and 3′-UTR, 3′-untranslated region. (**d**) A viral vector based on DNA containing the bean yellow dwarf virus (BeYDV) genome is schematically depicted. NPT II, neomycin phosphotransferase II gene conferring resistance to kanamycin; L, long intergenic region (LIR) in the BeYDV genome; TEV 5′-UTR, 5′-untranslated region the from tobacco etch virus (TEV); VSP 3′UTR, 3′-untranslated region from the soybean *vspB* gene; S, short intergenic region (SIR) from the BeYDV genome; TMV 5′-UTR, 5′-untranslated region from TMV; rbcS 3′-UTR, 3′-untranslated region from the pea *rbcS* gene; C2/C1, sequences encoding the replication initiation proteins Rep and RepA from the BeYDV genome; T, transcription terminator; and RB and LB, right and left borders of the Ti-DNA. The arrow points at the transcription direction from the L-promoter for the C1/C2 genes

Unlike non-replicating vectors, vectors based on the genomes of RNA-containing viruses show the ability to compete with one another for the sites of the formation of "viral factories," ultimately leading to the displacement of one vector by another in the replication and translation process (Giritch et al. 2006). Therefore, using vectors based on the genome of only one virus, such as TMV, it is not possible to achieve equimolar accumulation of the antibody light and heavy chain in the plant. It is necessary to choose a pair of viruses that do not compete in nature. Example of such a pair is TMV and PVX (Giritch et al. 2006). These vectors not only do not compete in the synthesis of antibody chains but also stimulate reproduction of each other.

In an attempt to solve the problem of viral vector competition and achieve an equimolar ratio of heavy and light chains, Hamorsky and colleagues (Hamorsky

et al. 2013) developed an approach in which a single vector is introduced into the cell. This vector encodes a polypeptide consisting of both antibody chains linked with an amino acid sequence (Zhang et al. 2011) recognised by the cellular endogenous Kex2p-like peptidase localised in the Golgi apparatus (Jiang and Rogers 1999) (Fig. 24.2c). This approach was used for the production of P-TMA VRC01 in *N. benthamiana*, which is intended for the treatment and prevention of AIDS by binding to the CD4 site. The P-TMA VRC01 is synthesised in leaf cells from a single viral vector based on the genome of cruciferous-infecting tobamovirus (Marillonnet et al. 2005). The vector encoded P-TMA VRC01 as a single polypeptide containing light and heavy chain fusion sequences, as well as a recognition site for the kex2p-like protease (Zhang et al. 2011). During maturation, this polypeptide was cut by an endogenous kex2p-like protease from *N. benthamiana* to form mature light and heavy chains that were assembled into full-sized antibody. The authors showed that it is possible to achieve maximum level of P-TMA accumulation (150 mg/kg of fresh leaf material) on the 5th-7th day post inoculation of the leaves with this method. However, the disadvantage of this method is the formation of a certain amount of unprocessed protein, which means that an additional purification process is essential.

For the production of another P-TMA for the prevention of AIDS, P2G12, it has been possible to successfully use a combination of two systems based on the modified RNA-2 from cowpea mosaic virus (CPMV) (Sainsbury et al. 2008) including (a) non-amplifying vectors under the control of the CaMV 35S promoter and (b) an amplifying system based on a deleted version of RNA-2 combined with a component based on CPMV RNA-1. This approach allowed production of up to 100 mg P-TMA/kg leaf fresh weight.

Vectors based on genomes of DNA-containing viruses can also be used for P-TMA production. Based on the Bean yellow dwarf virus (BeYDV) genome (Diamos et al. 2016; Huang et al. 2010), it was possible to create a vector directing the synthesis of both antibody chains against the Ebola virus glycoprotein GP1 from a single DNA molecule (Fig. 24.2d). The same vector was successfully used for the production of monoclonal antibody Rituximab at 1 mg/g leaf fresh weight (Diamos et al. 2016).

The stability of P-TMAs in the plant, including their resistance to plant proteases, is an important factor determining the antibody yield and integrity. Recently, a method for correcting the amino acid sequence of IgG has been proposed to remove potential cleavage sites recognised by plant proteases (Hehle et al. 2016). In mouse IgG produced in tobacco, cleavage sites were identified and then removed by site-specific mutagenesis. This genetic engineering promoted a significant reduction in IgG fragmentation and an increase in its yield when produced in tobacco plants. Another related strategy is based on the modification of the variable heavy chain (V_H) sequences when chimeric domains are created by mixing the HIV neutralising antibody 4E10 (low yield) and the tumour-specific antibody M12 sequences with a good yield in the plant (Zischewski et al. 2016). This approach increased the yield of antibodies up to 2000 μg/g FW 4 days after *N. benthamiana* leaf agroinfection, which exceeded the best index from the MagnICON system (Giritch et al. 2006).

Recently, the perspective use of methods to transiently express mAbs has also been considered (Ocampo and Petruccelli 2018). It is believed that the central vacuole is an appropriate compartment for the efficient production of P-TMAs because vacuolar sorting should be considered an alternative strategy to obtain high antibody yields.

24.5 Features of the P-TMA Carbohydrate Profile

Unlike mammals, plants produce oligosaccharides of lesser complexity and variety, as they do not synthesise, for example, branched and sialylated *N*-glycans. The high-mannose type of *N*-glycans in animals and plants contains five to nine mannose residues, whereas complex *N*-glycans in plants are structurally different from mammalian *N*-glycans (Bennett et al. 2018; Strasser 2016, 2014; Strasser et al. 2014) as follows: (a) in plant cell β-mannose from the glycan nucleus is replaced by β1,2 xylose, which is never found in mammalian *N*-glycans; (b) α1,3-fucose from nuclear proximal GlcNAc is found in place of α1,6-fucose of mammals; and (c) β1,3-galactose and α1,4-fucose bound to terminal GlcNAc in plant *N*-glycans form the Lewis A (Le^a) oligosaccharide structure, while in mammals β1,4-galactose is often combined with sialic acid (Beck et al. 2013; Gomord et al. 2010; Lerouge et al. 1998). These features of plant glycans are associated with their biogenesis.

Similar to in mammals, the biosynthesis of plant glycans occurs in the ER and Golgi apparatus and includes the activity of a variety of transmembrane enzymes, such as glycosyltransferases and glycosidases (Veit et al. 2015). The process takes place with the participation of dolichol and the synthesis of the dolichol-glycan precursor, which consists of dolichol phosphate (Dol-P) bound to a pre-assembled oligosaccharide consisting of 14 monosaccharides ($Glc_3Man_9GlcNAc_2$) that is conserved in all eukaryotes (Strasser 2016). N-glycosylation is initiated in the ER using the oligosacchar yltransferase (OST) transmembrane protein complex and the transfer of pre-assembled glycan ($Glc_3Man_9GlcNAc_2$) to asparagine (located in the amino acid sequence with the Asn-X-Ser/Thr motif, where X is any amino acid except proline) on the synthesised polypeptide chain. This stage is very important for plant life. The initial stages of the maturation process consist of the sequential removal of three Glc residues by glucosidases I and II and the production of $Glc_2Man_9GlcNAc_2$ and $Glc_1Man_9GlcNAc_2$, respectively. Chaperones calnexin and calreticulin recognise the proteins that carry $Glc_1Man_9GlcNAc_2$ and ensure proper folding. In the next step, glucose and the terminal α1,2-mannose from one of the glycan branches (Fig. 24.3) are removed by α-glucosidase II and α-mannosidase 3 (MNS3), respectively, to produce the glycan $Man_8GlcNAc_2$, which is transported to the Golgi apparatus to be modified by glycosyltransferases and glycosyl hydrolases for further *N*-glycan maturation. In plants, mannose trimming is performed by alpha-mannosidases (MNS1-MNS3), which act in the ER and *cis*-Golgi apparatus (Liebminger et al. 2009). The next stage of *N*-glycan maturation occurs in the medial compartment of the Golgi apparatus and is associated with the removal of α1-3- and α1-6-mannose by Golgi-α-mannosidase II (GM II) (Fig. 24.3).

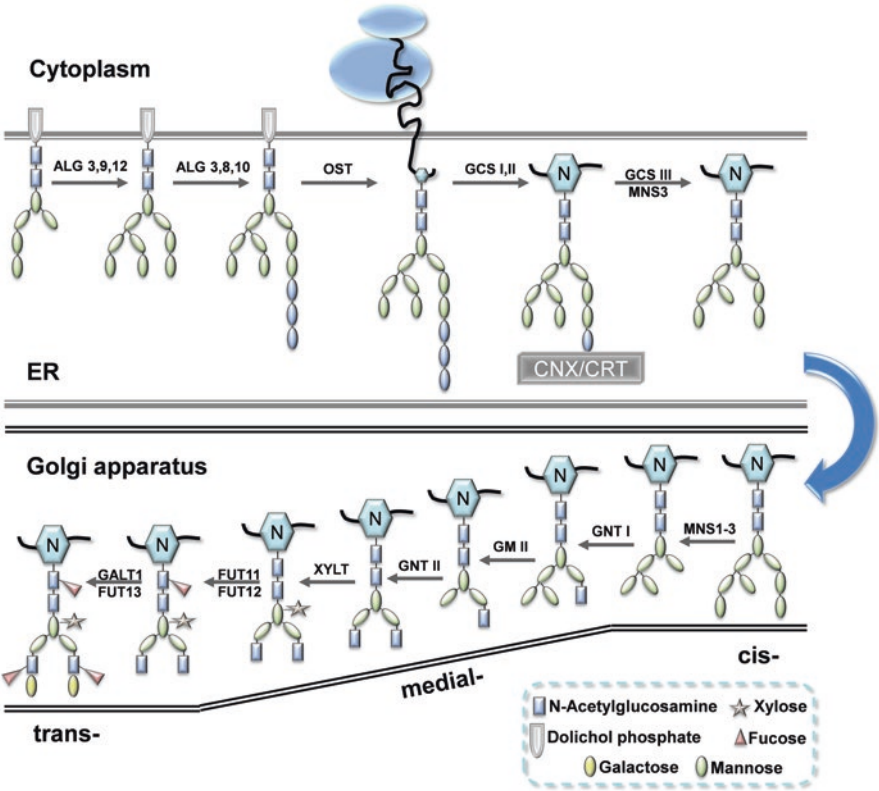

Fig. 24.3 Major stages in the formation of N-linked glycans in plants. The protein N-glycosylation process in the ER lumen includes the final steps of the asparagine-linked glycosyltransferases (ALG)-mediated formation of the dolichol phosphate-linked "glycan precursor" ($Glc_3Man_9GlcNAc_2$), from which the oligosaccharide is transferred to the asparagine of the protein target catalysed by oligosaccharyltransferase (OST). Next, Glc residue cleavage occurs in the ER catalysed by glucosidases (GCS), which is followed by further modification of the attached glycan in the Golgi apparatus. The cleavage of Man from high mannose forms is mediated by mannosidases (MNS1-3), than β1,2-N-acetylglucosaminyltransferase I (GNT I) catalyses the transfer of GlcNAc residue to α1,3-mannose with the further removal of α1-3- and α1-6-mannose by Golgi-α-mannosidase II (GM II) in the medial compartment of the Golgi apparatus, resulting in the hybrid N-glycan $GlcNAc_1Man_3GlcNAc_2$. GNT II catalyses the transfer of another GlcNAc residue to α1,6-mannose, which transitions from hybrid to complex N-glycans. The $GlcNAc_2Man_3GlcNAc_2$ glycan is further modified by β1,2-xylos yltransferase (XYLT) or the core α1,3-fucosyltransferases 11 and 12 (FUT11 and FUT12). Then, trans-Golgi β1,3-galactosyltransferase (GALT1) transfers the Gal residue to the terminal residues of GlcNAc through a β1,3-bond, resulting in the Galβ1-3GlcNAc structure. α1,4-fucosyltransferase 13 (FUT13) transports Fuc via an α1,4-bond to GlcNAc, completing the synthesis of the Lewis A oligosaccharide [Fucα1-4 (Galβ1-3) GlcNAc-R]

This enzymatic reaction is absolutely necessary for the formation of complex *N*-glycans. The resulting hybrid *N*-glycan $GlcNAc_1Man_3GlcNAc_2$ is a specific substrate for β1,2-*N*-acetylglucosaminyltransferase II (GNT II), which catalyses the transfer of another GlcNAc residue to α1,6-mannose, transitioning from hybrid to

complex N-glycans (Strasser 2016; Strasser et al. 2014; Varki et al. 2009). Alternatively, the $GlcNAc_2Man_3GlcNAc_2$ glycan can be transformed in the medial compartment of the Golgi apparatus with β1,2-xylos yltransferase (XYLT) or the core α1,3-fucosyltransferases 11 and 12 (FUT11 and FUT12), enzymes that fucosylate the core region of the biantennary glycan.

This glycan consists of a branching heptasaccharide and two disaccharide antennaes. The XYLT and FUT11/FUT12 transferases compete with one another for the same glycan substrates. The final steps in *N*-glycan modification occur in the *trans*-Golgi and are performed through the participation of β1,3-galactosyltransferase (GALT1) and α1,4-fucosyltransferase 13 (FUT13) (Fig. 24.3), which create the Lewis A [Fucα1-4 (Galβ1-3) GlcNAc-R] oligosaccharide structure. In the first step, GALT1 transfers a Gal residue through a β1,3-bond to the terminal residues of GlcNAc, which leads to the synthesis of the Galβ1-3GlcNAc structure. In the second step, FUT13 transports Fuc via an α1,4-bond to GlcNAc, completing the synthesis of the Lewis A oligosaccharide. These complex *N*-glycans are absent in mammals and can therefore cause unwanted immune responses when administered to humans (Dingjan et al. 2015). Interestingly, *A. thaliana* mutants deficient in the GALT1 and FUT13 enzymes lack the Lewis A oligosaccharide, and this does not affect the growth and development of plants (Strasser et al. 2007b).

Because plant and animal cells have similar mechanisms for gene expression, protein synthesis, and post-translational modification (Alberts et al. 2002), the expression of plant genes in an animal cell (Wada et al. 2017) and, conversely, animal genes in a plant cell are possible. Since the antibody is a secreted glycoprotein, the synthesised antibody chains, both in the animal and plant cells, undergo a maturation process involving glycosylation and the formation of disulfide bonds to assemble the antibody as a tetramer (Dorokhov et al. 2016). Similar to cultured human and animal cells, plants can perform post-translational modification of antibodies, including (a) disulfide bond formation for antibody assembly and (b) post-translational *N*-glycosylation, which is essentially the same as that occurring in mammalian cells (Strasser 2016). The P-TMA Asn297-bound glycan is mainly a glycan of the GnGnXF complex type (Strasser et al. 2008, 2009) according to glycan nomenclature (http://www.proglycan.com/protein-glycosylation-analysis/nomenclature). The core region of the P-TMA GnGnXF type Asn297-linked glycan contains α1,3-fucose and β1,2-xylose residues, which, in addition to the absence of terminal galactose and sialic acid (Strasser et al. 2007a), distinguishes P-TMA from human IgG (Fig. 24.4); however, as an example in the case of plant biosimilars for anticancer antibodies, it does not make them less active compared to the recombinant human or humanised IgG in suppressing the proliferation of cancer cells (Komarova et al. 2011, 2017b).

If we compare P-TMA with TMA, then a high homogeneity of the P-TMA carbohydrate profile is detected, which is a characteristic required by the regulatory rules. Indeed, if the P-TMA belongs to two types (GnGnXF and MMXF), the TMA preparation obtained in the culture of CHO cells contains five to seven Asn297-linked glycan structures (Strasser et al. 2008, 2009). For example, using a liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI-MS) method

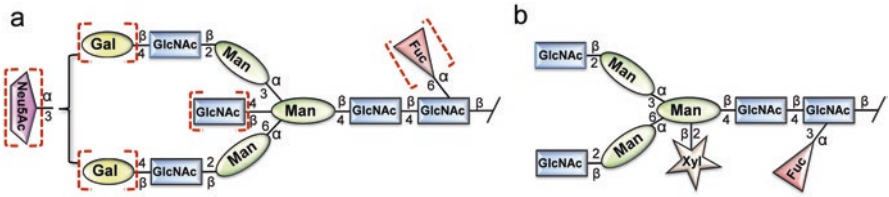

Fig. 24.4 Structure and variants of N-linked glycans produced in plants and mammalian cells and attached to the IgG heavy chain. (**a**) Glycans typical for human IgG and IgG produced in the CHO cell line are of the GnGn type and may contain α1,6-fucose, a terminal galactose and sialic acid; the addition of bisecting GlcNAc, which is not originally detected in CHO cells-produced IgG, prevents core fucosylation. (**b**) The main type of P-TMA N-linked glycan, GnGnXF, is characterised by the presence of plant-specific α1,3-fucose and β1,2-xylose

an anti-HIV 2G12 produced in a CHO cell culture was found to contain a mixture of structures including glycans, such as (a) galactose-free (GnGnD), (b) containing one (AGnF or GnAF) or (c) two (AAP) terminal galactose residues, as well as minor amounts of glycopeptides with terminal sialic acid (NAAF, NaNaF). Additionally, all the glycoforms found contained a fucose residue α1,6-bound to the GlcNAc closest to Asn297 (Strasser et al. 2008).

Asn297-linked P-TMA glycan is a specific plant glyco-epitope that is absent in mammals, although approximately 50% of people can detect antibodies to it in their blood (Bardor et al. 2003). Evaluation of the immunogenicity of the P-TMA Asn297-linked glycan gave inconsistent results in animal studies. In the first approach, mice immunised with murine IgG obtained in transgenic tobacco did not develop antibodies directed against either the protein or the glycan portion of the recombinant IgG (Chargelegue et al. 2000). In a different approach, rabbits were immunised with the P-TMA preparation P2G12 and antibodies to xylose and fucose were detected for the Asn297-linked glycan. Moreover, it turned out that IgE from patients with allergies are able to react with P2G12 in a Western blot (Jin et al. 2008).

An evaluation of the biosafety of immunogenic P-TMA for humans was recently carried out using a haemocyanin-conjugated idiotypic vaccine produced in a plant for the purposeful treatment of B-cell follicular lymphoma (Tusé et al. 2015). A personal vaccine was produced in the leaves of *N. benthamiana* plants agroinfiltrated with a genetic construct encoding isolated Fab-sites for light and heavy chains and fused to the constant domains of normal human IgG1. All recombinant anti-idiotypic antigens used in this study were glycoproteins containing oligomannose glycans such as MMXF and GnGnXF type glycans. After administration, this personalised idiotypic vaccine induced idiotypic antibodies that inhibited tumour growth in 9 of 11 patients. At the same time, none of the patients showed serious side effects associated with vaccination, i.e., administration of antibodies with typical P-TMA glycosylation (Tusé et al. 2015). Nevertheless, despite the safety of plant antibodies demonstrated in these studies, there are still concerns that certain plant glycans may be immunogenic to humans (Bosch et al. 2013), which in turn dictates the need for P-TMA "humanisation".

The study of the role of carbohydrate residues in the functioning of human IgG and TMA obtained in the culture of animal cells showed that the biological activity of the antibody depends on the particular carbohydrate residue contained in the *N*-glycan (Niwa and Satoh 2015). For example, the fucose residue attached to the *N*-glycan core via the α1,6-bond reduces the binding efficiency of the Fc fragment to the Fcγ IIIA receptor (FcγRIIIA), and, as a consequence, the activation of ADCC. Defucosylation of TMA Asn297-glycan multiplies the degree of its binding to FcγRIIIA, and thus increases the effector function of ADCC (Ferrara et al. 2006; Jefferis 2009).

In P-TMA, fucose is attached to the Asn297-linked glycan core via an α1,3-bond and is also capable of reducing the efficiency of the interaction of the Fc fragment with FcγRIIIA. Elimination of fucose residues from the constant part of the antibody makes plant antibodies more effective at stimulating ADCC (Holtz et al. 2015; Zeitlin et al. 2011).

Due to the differences in the glycosylation profile of plant-made antibodies from those produced in animal cells, the researchers realised the need to develop methods and create conditions under which the obtained P-TMA could have a carbohydrate profile that is as close as possible to human IgG. The "humanisation" of the producer plant have the following aims: (1) the elimination of the P-TMA immunogenicity, (2) the acquisition by the plant of human genes and the ability to synthesise P-TMA with the carbohydrate profile of human IgG, and (3) the suppression of β1,2-xylosylation and α1,3-fucosylation of the Asn297-linked glycan core portion. At the present, the following two methods for "humanising" P-TMA have been proposed: (a) subcellular targeting in the ER using a signal sequence (HDEL/KDEL) and (b) glycomodification, where genomic engineering eliminates unwanted glycosylation enzyme genes and some genes providing the synthesis of "humanised" *N*-glycans are added.

Subcellular targeting is historically an earlier technique that could help change the glycosylation profile. With the HDEL/KDEL signal sequence, the antibody is retained in the ER and thus the Golgi apparatus is not involved in antibody maturation, which prevents the addition of β1,2-xylose and α1,3-fucose to the Asn297-bound glycan core portion. With this technique, several KDEL-containing P-TMAs were synthesised in transgenic tobacco plants. One is cPIPP, a hybrid (mouse/human) IgG1 binding the β-subunit of the human chorionic gonadotropin and containing the KDEL sequence at the C-terminal end of both antibody chains (Sriraman et al. 2004). The analysis showed that cPIPP contained only high-mannose *N*-glycans, which is typical for glycoproteins that did not undergo maturation and trimming in the Golgi apparatus. In contrast, cPIPP lacking the KDEL sequence contained complex glycans including core-bound α1,3-fucose and β1,2-xylose. Similar work has also been done with KDEL-containing P-TMA against the surface antigen of the hepatitis B virus (HBsAg) (Triguero et al. 2005). Although the KDEL sequence was also located in the C-terminal part of both chains, approximately 10–20% of the antibodies contained glycans of complex types. The differences in these results are explained by the conformational features of the Fc domain and by the "availability" of the KDEL sequence (Petruccelli et al. 2006).

The second method, the *N*-glyco engineering of producer plants, developed in recent years, has brought more successes in the creation of glyco modified P-TMAs (Bosch et al. 2013; Loos and Castilho 2015). This was facilitated by the fact that, in general, plants tolerate a change in the protein glycosylation profile. Thus, the elimination of complex *N*-glycans, suppression of β1,2-xylosylation, and α1,3-fucosylation of the Asn297-linked glycan core portion generally does not result in significant changes in the growth and development of *A. thaliana* plants (Dicker et al. 2016; Strasser 2016; Strasser et al. 2004) or *N. benthamiana* (Bennett et al. 2018; Nagels et al. 2011; Strasser et al. 2008). This general tolerance for N-glyco engineering is a prerequisite for humanising the biosynthetic pathways associated with plant *N*-glycosylation.

Elimination of plant glycans in P-TMAs is aimed primarily at avoiding the processes of β1,2-xylosylation and α1,3-fucosylation at the P-TMA Asn297-bound glycan core region, i.e., those processes that are absent in humans. The reality of this strategy was proven in *A. thaliana* plants, in which the genes encoding XYLT and FUT were inactivated (Strasser et al. 2004). The work was carried out in several stages. In the beginning, *xylt* plants were obtained, in which only the *A. thaliana XYLT* gene was knocked out. In the next step, *futa* and *futb* plants were successively obtained, and then they were crossed and *fuct* plants were selected among the offspring, in which both genes were knocked out. During the last stage, the plants were crossed and *xylt/fut* progeny were obtained, in which the synthesis of XYLT and FUT was suppressed. This knockout had no effect on plant viability, but abolished the synthesis of a potentially immunogenic *N*-glycan. Instead, structures with two terminal residues of GlcNAc ($GlcNAc_2Man_3GlcNAc_2$) were synthesised preferentially.

Subsequently, this glycomodification technology was applied to other plant species, including *N. benthamiana*, the main producer of P-TMAs. Using the knockout of the gene by RNA-interference (RNAi), an *N. benthamiana ΔXTFT* double mutant (Strasser et al. 2008) was obtained. Using the example of the antibody P2G12, glycoforms were studied and it was shown that the xylose and α1,3-fucose residues were absent in the Asn297-bound glycan. Thus, the plant-derived P2G12 antibodies did not differ from 2G12 obtained in CHO cells, and both had the ability to bind antigens and neutralise HIV (Strasser et al. 2008), though the plant antibody had a more pronounced ability to stimulate ADCC (Forthal et al. 2010).

N. benthamiana ΔXTFT plants proved to be a convenient tool for the "humanisation" of P-TMA Cetuximab, the original variant of which was obtained in the culture of mouse SP2/0 cells (Castilho et al. 2015). The problem is that Cetuximab, which is a chimeric antibody (mouse/human), contains *N*-glycans in both the Fc- and Fab-regions. Cetuximab produced in wild-type plants contained xylose and α1,3-fucose in the *N*-glycan core portion, but its synthesis in *ΔXTFT* plants resulted in the removal of these residues. Another alternative method for removing β1,2-xylose and α1,3-fucose from *N*-glycan is known. The *N. benthamiana ΔGMDΔXYLT* double mutant with knocked out GTP-D-mannose 4,6-dehydratase and β-1,2-XYLT genes (Matsuo et al. 2014) also provided the synthesis of P-TMA devoid of plant-type Asn297-linked glycans.

The introduction of mammalian genes into plant cells allowed the synthesis of "humanised" *N*-glycan with the GlcNAc residue in the mannose fork of the Asn297-glycan core. From TMA studies, it is known that the appearance of GlcNAc in the mannose fork of the Asn297-glycan core inhibits the further addition of fucose, and this entails an increase in the effectiveness of the ADCC function of the antibody (Ferrara et al. 2006). Thus, increased expression of β1,4-N-acetylglucosaminyltransferase III (GNT III) may contribute to an increase in this effector function of the antibody. The problem is that plants do not have GNT III, thus cannot contain additional GlcNAc in the mannose fork of the *N*-glycan core (Fig. 24.4). The introduction of mammalian GNT III into the plant cell could overcome this "deficiency". Indeed, the introduction of rat (Frey et al. 2009) or human GNT III (Rouwendal et al. 2007) in tobacco led to the formation of not only the desired *N*-glycan structure in P-TMA but also unusual glycans. For example, this technique resulted in the appearance of terminal GlcNAc residues, which are unusual for wild-type plants (Rouwendal et al. 2007). The use of chimeric mammalian GNT III containing the signal sequence that sent it to the *trans*-Golgi apparatus resulted in the formation of *N*-glycans with GlcNAc in the mannose fork and the lack of the fucose residue (Bosch et al. 2013).

The end galactose in the human IgG Asn297-glycan (a) participates in the binding of the Fc fragment to FcγRIIIA (Houde et al. 2010) and (b) is an acceptor substrate for the formation of the final Asn297-linked glycan complex in the *trans* department of the Golgi apparatus. Plant *N*-glycans do not contain a terminal galactose because the β1,4-galactosyltransferase (GalT) enzyme is absent. The introduction of the sequence encoding the human GalT into the *N. benthamiana* plant genome makes it possible to obtain transgenic plants capable of β1,4-galactosylating the terminal residues of *N*-glycans (Strasser et al. 2009). Moreover, it has been shown that bi-galactosylated anti-HIV P-TMAs have an enhanced ability to neutralise the virus.

The terminal sialylation of Asn297-linked glycan in human IgG is the final stage of *N*-glycosylation. The sialylated IgG1 content in healthy human blood plasma varies from 2% to 5%. Sialylated TMA has an anti-inflammatory effect and increases the resistance of TMA to proteolysis in the patient's bloodstream. In plants, sialylated proteins are not detected (Zeleny et al. 2006) because there is no CMP-Neu5Ac biosynthesis and delivery system to the Golgi apparatus or a sialyltransferase that transfers Neu5Ac to the terminal galactose of Asn297-linked glycans (Bosch et al. 2013).

Therefore, to sialylate P-TMAs Asn297-linked glycan, it is necessary to introduce human genes into plant cells to ensure the synthesis of missing enzymes (Castilho et al. 2010; Jez et al. 2013). The feasibility of this approach was proven in *N. benthamiana* plants transfected with animal and human genes to incorporate the entire sialylation process, including activation and transport of Neu5Ac to terminal galactose (Castilho et al. 2010). The introduction of the mouse gene encoding the UDP-GlcNAc-2 epimerase/N-acetylmannosamine kinase, the CMP-Neu5Ac carrier; the human genes encoding the phosphate-Neu5Ac synthase, the CMP-Neu5Ac synthase, and GalT; and the rat gene encoding the α2,6-sialyl transferase resulted in sialylation of up to 80% of N-glycans (Jez et al. 2013).

It is known that the lack of Asn297-linked glycan reduces the ability of TMA to activate ADCC. Thus, non-glycosylated Cetuximab is unable to bind FcγRI and FcγRIIIa, and, as a consequence, is unable to activate ADCC (Patel et al. 2010). Rituximab and Trastuzumab produced in CHO cell culture usually contain less than 1% of non-glycosylated forms or do not contain them at all. However, when obtaining P-TMA Trastuzumab-P and Rituximab-P, the proportion of non-glycosylated forms increases to 20–30% (Jarczowski et al. 2016). A method that introduces amino acid substitutions into the consensus sequence of the Asn297 site has been shown to reduce the content of the non-glycosylated forms to 5–7% (Jarczowski et al. 2016).

24.6 Antiviral and Antibacterial P-TMA

24.6.1 Human Immunodeficiency Virus (HIV)

Infection caused by human immunodeficiency virus (HIV)-1 and HIV-2, which destroy the human immune system, leads to increased susceptibility to a wide range of infections, cancer and other diseases (Engelman and Cherepanov 2012). HIV continues to be a major global health problem worldwide. It is known that the use of drugs (ointments and creams) containing anti-HIV TMA as a microbicide with topical application (vaginal, anal) prevents sexually transmitted infection (Morris et al. 2014). The need for a microbicide is increasing every year and is now estimated at 5 tons per ten million people. Currently, HIV-neutralising P-TMA has been obtained using (a) stably transformed maize (Rademacher et al. 2008), *A. thaliana* (Schähs et al. 2007) and tobacco (Ma et al. 2015) and (b) transiently expressing *N. benthamiana* (Hamorsky et al. 2013; Sainsbury et al. 2010). The local application of the P-TMA P2G12 promises to provide a relatively inexpensive and safe way to protect people. The safety of this method has already been confirmed by a first in-human phase I clinical trial (Ma et al. 2015). P2G12 obtained in transgenic tobacco did not cause any side effects or undesirable immunological effects after vaginal administration to 11 female volunteers (aged 18–50 years) as a microbicide (Ma et al. 2015).

With the widespread use of P2G12, an expression system is needed that provides low-cost P-TMA production in large quantities. Rice grains containing P2G12 can be such a source. A detailed study of the properties of the P2G12 antibody accumulating in the endosperm of rice grains (Vamvaka et al. 2016) showed that its heavy chain is not glycosylated. Nevertheless, the heavy and light chains were assembled into functional P2G12 with more potent HIV neutralising activity than P2G12 obtained in other plant systems and characterised by the presence of typical high-mannose glycans or complex-type glycans. It was also possible to obtain rice that simultaneously accumulates three functional HIV-neutralising proteins (the monoclonal antibody 2G12 and the lectins griffithsin and cyanovirin-N) in the grains (Vamvaka et al. 2018).

Moreover, extracts from transgenic plant producing all three proteins showed enhanced in vitro binding to gp120 and synergistic HIV-1 neutralisation. It has been suggested that the production of HIV-1 microbicides in rice may not only reduce costs compared to traditional platforms, but may also provide functional benefits in terms of microbicidal potency (Vamvaka et al. 2018).

The lines of *N. benthamiana* ΔXTFT (Strasser et al. 2008) were also used to optimise and "humanise" the carbohydrate profile of P2G12. P-TMAs were obtained with an almost uniform carbohydrate composition for human N-glycans without xylose or α1,3-fucose in the core of Asn297-linked glycan (Forthal et al. 2010).

The prevention and control of AIDS when using P-TMA focuses on local application, and it is natural to consider the use of secretory immunoglobulin IgA (sIgA). sIgA is an oligomeric protein complex containing two molecules of IgA connected by a J-chain and a secretory component (Fig. 24.5a). Recently, it has become possible to obtain stably transformed tobacco and to create a transient expression system for sIgA products. Using a series of binary vectors from the pEAQ family (Sainsbury et al. 2009), four genes encoding the light and heavy chains, the J-chain and the secretory component were delivered to the leaves of *N. benthamiana* (Fig. 24.5b). Both systems produced sIgA P2G12 (Paul et al. 2014). The resulting sIgA protein complexes were absent in the apoplast but accumulated in intracellular compartments including the vacuoles. The maximum yield of P2G12 sIgA was 15 μg/g (transgenic tobacco) and 25 μg/g (agroinjected *N. benthamiana*) of raw green mass. After affinity chromatography, the purified antibody specifically bound to HIV gp140. Analysis of the carbohydrate composition revealed predominantly high mannose structures. An important advantage of P2G12 sIgA over P2G12 IgG is its higher stability in secretions from the vaginal mucosa, which implies its prospect in future uses (Paul et al. 2014; Westerhof et al. 2014).

24.6.2 Ebola Virus

Ebola haemorrhagic fever is a highly contagious disease that is prevalent in West African countries and causes a mortality rate greater than 70% in the population. People become infected by contact with infected animal tissues or by inhaling the aerosol of their excrement. Transmission from person to person occurs as a result of contact with infected blood or other body fluids. Infection causes damage to the vascular system, leading to bleeding in subcutaneous and internal organs. Ebola fever is caused by the small RNA-containing filovirus Ebola virus (EV). Modern methods of treatment are the creation of antibodies directed to the main virulence factor of EV, a transmembrane glycoprotein that participates in (a) penetration of EV into the host cell, (b) the development of cytopathic effects, and (c) induction of protective antibodies in infected patients.

A transient expression system in *N. benthamiana* ΔXTFT (Strasser et al. 2008) was effective in creating a "cocktail" of P-TMA MB-003 consisting of the human and chimeric (human/mouse) monoclonal antibodies c13C6, h13F6 and c6D8 (Olinger et al. 2012). Infected monkeys (rhesus macaques) were fully protected after

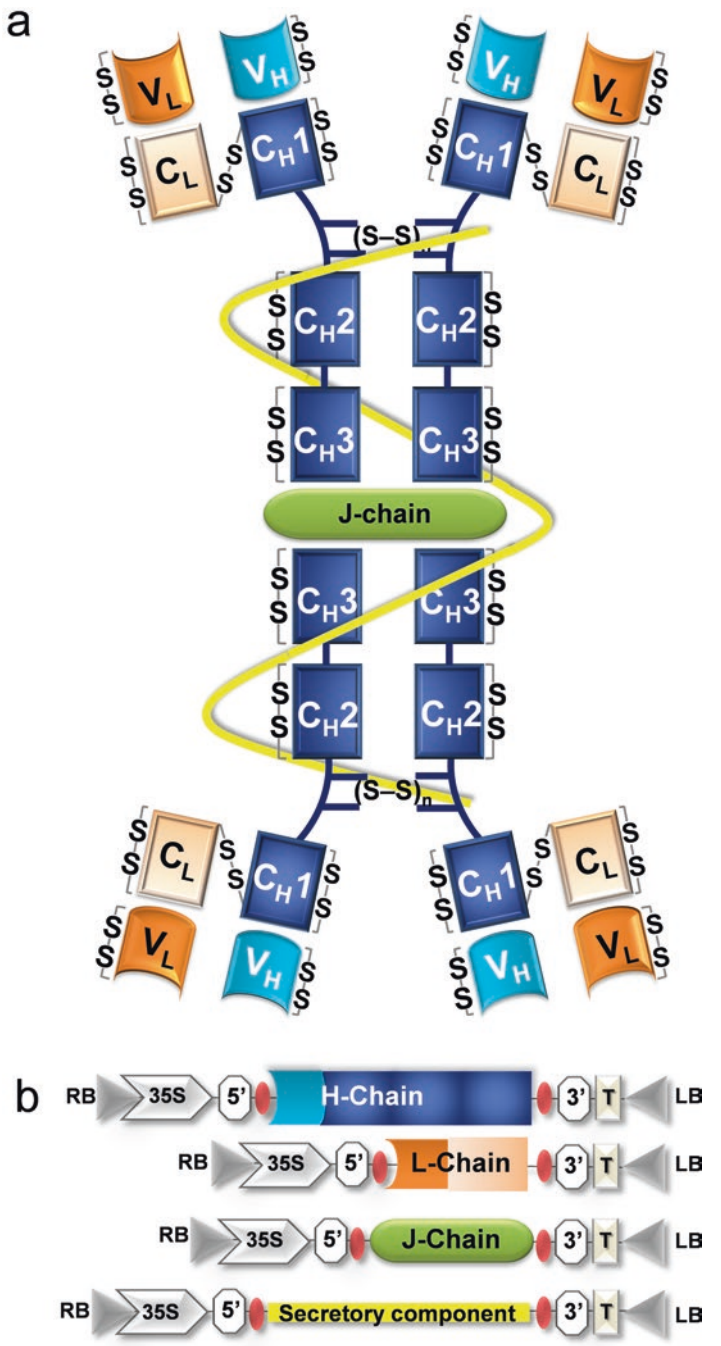

Fig. 24.5 Secretory immunoglobulin sIgA. (**a**) The schematic representation of the main structural elements of sIgA. sIgA contains heavy (H-) and light (L-) chains together with the joining (J-) chain and secretory component. (**b**) Schematic representation of the pEAQ-family binary vectors used for sIgA production. 35S, 35S promoter from CaMV; CPMV 5′-UTR, 5′-untranslated region from the CPMV RNA-2; CPMV 3′-UTR, 3′-untranslated region from the CPMV RNA-2; T, transcription terminator; and RB and LB, right and left border of the Ti-DNA. Red ovals denote the recombination sites

injection of MB-003 intravenously 1 h after infection with a lethal dose of EV, and then again 4 and 8 days later. The MB-003 mixture was superior to the corresponding antibodies produced in CHO culture, and its components did not contain fucose residues in the Asn297-linked glycan core, which stimulated ADCC (Zeitlin et al. 2011). Another approach based on production of the *N. benthamiana* "cocktail" ZMapp (clones c13C6 from MB-003 plus humanised clones c2G4 and c4G7 from ZMAb) provided protection for Javanica macaques (Qiu et al. 2011, 2012, 2014).

With regard to testing drugs in humans, during the outbreak of Ebola haemorrhagic fever in West Africa from 2014 to 2015, seven patients were injected with ZMapp, although its safety had not previously been tested in humans. Five patients improved significantly and recovered, though their treatment was started 9 days after infection (Lyon et al. 2014; McCarthy 2014). Now, after successful emergency care for patients, systematic studies of biosafety and treatment effectiveness (Phase I/II) with ZMapp have been started.

Furthermore, anti-Ebola P-TMA can be used as a vehicle for creating a vaccine immune complex (Phoolcharoen et al. 2011). When using a geminiviral replicon, it was possible to create a complex in *N. benthamiana*. EV glycoprotein (GP1) was fused to the C-terminus of the heavy chain of a humanised 6D8 monoclonal antibody that specifically binds to the GP1 epitope. Co-expression of the gene encoding the heavy chain-GP1 fusion protein and the gene encoding the light chain of 6D8 resulted in the formation of immune complexes capable of stimulating the formation of anti-EV antibodies in mice (Phoolcharoen et al. 2011).

24.6.3 Junin Virus

The Junin virus (JV), which belongs to the group of arenaviruses, is the causative agent of Argentine haemorrhagic fever. Although the prevalence of JV is limited and mainly in Argentina, it tends to be transferred to other territories. Additionally, epidemiologists fear that JV can be used for bioterrorism purposes because the mortality rate without treatment is 20–30%. Treatment with immune serum for 8 days removes symptoms and reduces mortality to 1% (Enria et al. 2008).

In 1989, monoclonal antibodies reacting with the JV surface glycoproteins (Sanchez et al. 1989) were obtained. To obtain P-TMAs, genes encoding 3 clones of mouse-neutralising monoclonal antibodies were expressed in *N. benthamiana* ΔXTFT (Strasser et al. 2008) to obtain three anti-JV P-TMA clones (Zeitlin et al. 2016). The N-glycosylation profile indicated the presence of mainly GnGn glycoforms. The introduction of these P-TMA to guinea pigs, which are a model for the study of Argentine haemorrhagic fever, caused 100% protection from lethal doses of JV. Moreover, the therapeutic effects of the anti-JV P-TMA exceeded accepted medications (ribavirin, serum and others).

24.6.4 Human Respiratory Syncytial Virus

A common cause of severe lower respiratory infections in infants, young children, and elderly patients in long-term care settings is human respiratory syncytial virus (HRSV). Passive immunisation against HRSV is effective, as evidenced by the popularity of Palivizumab produced in the mouse NS0 cell culture, which is a humanised TMA based on IgG1 against the HRSV F protein. The high cost of this drug forces researchers to develop a plant production platform that allows for a 20-fold reduction in the cost of Palivizumab (Whaley et al. 2014).

Recently, the Palivizumab biosimilar P-TMA (Palivizumab-P) was obtained (Whaley et al. 2014; Zeitlin et al. 2013) in *N. benthamiana* ΔXTFT plants (Strasser et al. 2008). A comparison of the carbohydrate profile showed that Palivizumab-P has homogeneous N-glycosylation with the predominance of GnGn-glycans and a small proportion of high-mannose variants. In contrast, the Palivizumab *N*-glycans are heterogeneous, and mostly contain fucose (Hiatt et al. 2014). When Palivizumab-P was compared with commercial Palivizumab, both drugs showed similar prophylactic and therapeutic efficacy in a cotton rat model (Zeitlin et al. 2013).

24.6.5 Flaviviruses

The *Flavivirus* genus belongs to the family of *Flaviviridae*, which consists of more than 70 viruses including insect-specific flaviviruses. The majority of flaviviruses are transmitted by mosquitos or ticks (Calzolari et al. 2016). Many flaviviruses are important human pathogens, including Dengue virus (DENV), West Nile virus (WNV), and Zika virus (ZIKV). WNV is most often transmitted to humans through bites from infected mosquitoes from the genus *Culex* (*Culex pipien*s) and causes fever, encephalitis or meningitis. There is no vaccine against WNV; however, immunotherapy with the use of antibodies is considered promising. Dengue fever (DF) is a widespread disease caused by DENV and is endemic to the tropical areas of Africa, Southeast Asia and South America. It is a mosquito-borne illness spread primarily by the urban-adapted species *Aedes aegypti.* ZIKV is closely related to the four serotypes of DENV and its infection has been linked to the development of severe foetal abnormalities that include microcephaly and Guillain–Barré syndrome in adults. In 2015, over 1.5 million people were infected with ZIKV in Brazil. Most neutralising antibodies isolated from WNV-, DENV-, or ZIKV-infected human or mouse sera have been mapped to three domains in E glycoprotein (Beltramello et al. 2010). However, research focused on immunotherapy for this infection showed that for an antibody to be effective and therapeutic against flavivirus infection, it must be able to neutralise the virus and preferably induce ADCC and complement-dependent cytotoxicity (CDC), but not induce antibody-dependent enhancement (ADE) of infection, which poses a potential risk to predispose treated patients to a more severe secondary infection. Human therapeutics against flaviviruses are largely

dependent on the elimination of the ADE biosafety concerns and the speed and economics of antibody production. This explains the researchers' desire to create a system of P-TMA against WNV, DENV and ZIKV (Sun et al. 2017), as plants may provide solutions to overcome both the biosafety and economic challenges of flavivirus therapeutic development. At the present, the monoclonal antibody E16 against WNV E glycoprotein has been isolated and its humanised version, hE16, was obtained. Both antibodies demonstrated a curative effect after administration to infected mice (Oliphant et al. 2005). Using various vector systems, hE16 was synthesised in the leaves of *N. benthamiana* and salad with yields of 800 and 260 mg per kilogram of fresh biomass, respectively (Lai et al. 2010). When using *N. benthamiana* ΔXTFT plants, glyco-modified hE16 variants were also obtained, which showed high neutralising activities (He et al. 2014; Lai et al. 2014). In general, it is believed that an anti-WNV P-TMA may become an effective beneficial agent for WNV immunotherapy (Yusibov et al. 2016). To neutralise all four serotypes of DENV, E60 TMAs were obtained (Pierson et al. 2007). However, mammalian cell-produced TMA E60 exhibits ADE activity, which renders it inefficacious in vivo, as treated animals become more susceptible to developing more severe diseases during secondary infection. However, if TMA E60 is produced in *N. benthamiana* (P-TMA E60), Fc gamma receptor-expressing human cells exhibited reduced ADE activity (Dent et al. 2016). Thus, a plant system for E60 production allows minimising ADE, which may lead to the development of safe and highly efficacious antibody-based therapeutics against DENV and other ADE-prone viral diseases. (Dent et al. 2016). P-TMA against ZIKV E glycoprotein epitopes have also been created in *N. benthamiana* plants where they are assembled efficiently. Moreover, some of them had potent neutralising activities against ZIKV as well as DENV (Sun et al. 2017).

24.6.6 Anthrax

Anthrax is a dangerous infectious disease caused by the gram-positive, anaerobic, spore-forming bacterium *Bacillus anthracis.* The main virulence factor is the 83-kDa protective antigen (PA), which forms exotoxins that kill cells. PA is the main target for the creation of vaccines and TMAs, as it induces the formation of specific neutralising antibodies in the body. P-TMA against anthrax is considered, first of all, as a means of combating bioterrorism. P-TMA against PA was obtained in *N. benthamiana* plants. The ability of P-TMA against PA to neutralise toxin activity both in vitro and in vivo was demonstrated at a level comparable to the original TMA (Hull et al. 2005).

The non-glycosylated form of P-TMA against PA proved to be more effective in protecting mice that received a lethal dose of *B. anthracis* (Mett et al. 2011). When testing primates, the effectiveness of the non-glycosylated form of P-TMA against PA was even higher. It is believed that the non-glycosylated form of P-TMA against PA should be used to prevent the inhalation of *B. anthracis* spores.

24.6.7 Botulism

The botulinum neurotoxin produced by the anaerobic bacterium *Clostridium botulinum*, for example in spore-contaminated canned foods, causes lethal damage to the nervous system. Treatment of botulism involves the administration of an antitoxin as soon as possible after diagnosis. Transgenic tobacco producing single-chain variable fragments (scFvs) based on antibodies that neutralise the toxin have been obtained, (Almquist et al. 2006), and calculations showed that a plantation (1–2 ha) of this tobacco allows the accumulation of up to 4 kg of antitoxin, which is sufficient to produce 1 million therapeutic doses.

24.7 Anticancer P-TMA

24.7.1 Rituximab-P

As a sales leader among anticancer drugs, Rituximab (MabThera®, Rituxan®), which interacts with CD20, provides effective treatment for lymphoma and leukaemia. The original preparation was obtained from CHO cell culture. The production of Rituximab in plants (Rituximab-P) was established by Caliber Biotherapeutics (USA) using a two-vector (TMV and PVX) approach (Giritch et al. 2006), which allowed obtaining up to 650 mg of antibodies per kg of fresh *N. benthamiana* leaf weight (Holtz et al. 2015). Rituximab-P, which is a plant biosimilar, did not differ from the original Rituximab in its ability to activate ADCC, while the glycomodified P-TMA, which is similar to the glycomodified TMA Obinutuzumab (Gazyva®) (Evans and Clemmons 2015), had a two to tenfold superiority over the original drug (Holtz et al. 2015). Another approach to obtain glycolmodified Rituximab-P with uniform Gal2GlcNAc2Man3GlcNAc2 N-linked glycan lacking α1,6- or α-1,3-fucose and β-1,2-xylose residues was developed (Bennett et al. 2018). It is based on an in vivo deglycosylation followed by an in vitro chemoenzymatic glycosylation. The resulting antibody was shown to have CD20-binding affinity comparable to that of Rituximab but demonstrated significantly enhanced ADCC response in vitro (Bennett et al. 2018).

It has recently been shown that an immunocytokine (2B8-Fc-hIL2) obtained by fusing an anti-CD20 scFv-Fc antibody derived from Rituximab to human interleukin 2 (hIL-2) can be efficiently produced in *N. benthamiana* plants (Marusic et al. 2016). Moreover, the glyco-engineered recombinant molecules also exhibit strongly improved ADCC and CDC (Marusic et al. 2018).

24.7.2 Trastuzumab-P and Pertuzumab-P

The original preparation of Trastuzumab (Herceptin®), which is intended for the treatment of HER2-positive breast cancer, is obtained from CHO cell culture. The plant biosimilar (Trastuzumab-P) was obtained from stably transformed tobacco (Garabagi et al. 2012) and in a transient expression system in *N. benthamiana*

(Grohs et al. 2010; Komarova et al. 2011). Trastuzumab-P is capable of (a) binding the HER2/neu oncoprotein on the surface of cancer cells, (b) inhibiting cancer cell proliferation and (c) inhibiting tumour growth in mice with xenografts of human SK-BR-3 cancer cells (Komarova et al. 2011). However, there is currently no data on the biological activity of glycomodified Trastuzumab-P in the literature.

We recently created the first pertuzumab plant biosimilar (PPB) and investigated the composition of its Asn297-linked glycan in comparison with a trastuzumab plant biosimilar (TPB) (Komarova et al. 2017b). Both biosimilars were produced in wild-type (WT) *Nicotiana benthamiana* plants (PPB-WT and TPB-WT) and transgenic ΔXTFT *N. benthamiana* plants with *XT* and *FT* gene knockouts (PPB-ΔXTFT and TPB-ΔXTFT). Western blot analysis with antibodies against α1,3-fucose and xylose, as well as testing using peptide-N-glycosidase F, confirmed the absence of α1,3-fucose and xylose in the Asn297-linked glycan of PPB-ΔXTFT and TPB-ΔXTFT. Peptide analysis followed by the identification of glycomodified peptides using mass spectrometry showed that PPB-WT and TPB-WT Asn297-linked glycans are mainly complex type GnGnXF. Analysis of the TPB-ΔXTFT total carbohydrate content indicates the possibility of changing the composition of the carbohydrate profile not only for the Fc but also for the Fab portion of the antibody produced in transgenic *N. benthamiana* ΔXTFT plants. The study of the antigen-binding capacity of the biosimilars showed that the absence of xylose and fucose residues in the Asn297-linked glycans did not affect the ability of the glycomodified antibodies to interact with HER2/neu-expressing cancer cells.

24.7.3 Bevacizumab-P

Bevacizumab, a TMA targeting vascular endothelial growth factor (VEGF), has been widely used in clinical practice for the treatment of multiple cancers (Ferrara et al. 2005). Recently, the first plant-derived Bevacizumab (Bevacizumab-P) obtained in transgenic rice callus was reported (Chen et al. 2016). Enzyme-linked immunosorbent assay analysis confirmed that the rice-derived Bevacizumab-P was biologically active. Moreover, Bevacizumab-P and a commercial Bevacizumab (Avastin) were shown to have similar binding affinity to hVEGF. Thus, rice callus-produced Bevacizumab-P might potentially be used as a cost-effective biosimilar molecule in future cancer treatments.

24.7.4 Idiotypic Vaccine for the Treatment of Non-Hodgkin's Lymphoma (NHL)

Rituximab is usually used to treat NHL, but 30–50% of patients have resistance, which can be overcome by an anticancer vaccine based on individual idiotypic antibodies. Anti-idiotypic antibodies are human antibodies against the hypervariable regions of antibodies produced by the organism itself. The NHL idiotype is a unique tumour-specific antigen of the surface of malignant B-lymphocytes that includes

variable regions from the IgG heavy and light chains. The creation of an individual vaccine began by selecting a tumour site from patients and identifying the nucleotide sequence encoding the variable (Fv) regions in the tumour-specific antigen heavy and light chains. The cDNA encoding the corresponding scFv fragment was then placed into a vector based on the TMV genome. The infection of tobacco with this vector made it possible to obtain an idiotypic vaccine against NHL, which consisted of a short polypeptide corresponding to Fv (McCormick et al. 1999).

The feasibility of this technology was subsequently confirmed by a study of 16 patients with NHL. It was possible to create 16 individual vaccines and confirm their safety and immunogenicity (McCormick et al. 2006). Subsequently, the technology for creating a vaccine for the treatment of NHL was improved (Bendandi et al. 2010). The nucleotide sequence encoding the heavy and light chain segment Fv for the tumour-specific antigen was supplemented with human IgG1 constant regions, resulting in a full-length antibody that was synthesised in *N. benthamiana* using a two-vector system (Giritch et al. 2006). This approach was used to create an individual vaccine for 11 patients with NHL, i.e., 11 individual vaccines were prepared. The creation of each vaccine took less than 12 weeks (from biopsy to vaccine) due to the high expression levels in the plant (0.5–4.8 g per kilogram of fresh leaf tissue). In the next step, the idiotypic vaccine was chemically conjugated to the carrier protein haemocyanin and administered to patients with granulocyte-macrophage colony-stimulating factor in the form of six consecutive subcutaneous injections. Of the 11 patients who received the vaccine, 9 (82%) had an idiotype-specific cellular and humoral immune response. None of the patients showed serious side effects associated with vaccination (Tusé et al. 2015).

24.8 Techno-economic Analysis of Plant-Based Platform for P-TMA Production

Approximately 10 tons of diagnostic and therapeutic mAbs were manufactured in 2013, and, according to forecasts, demand could double by 2020 (Ecker et al. 2015). It is clear to experts that the use of mammalian cell cultures, which are today's gold standard for production, cannot meet these requirements (Shukla and Gottschalk 2013). Unlike mammalian cell fermentation platforms, plants are effectively living single-use bioreactors that can be scaled by simply sowing more seeds. Closely cropped tobacco in an open field (Stoger et al. 2014) and vertical farming units (VFUs) (Holtz et al. 2015) are capable of yielding from 182 t km^{-2} y^{-1} (open field production) up to 91,000 t km^{-2} y^{-1} (VFU) (Buyel et al. 2017). However, because of the low expression levels and consequently the low yield of P-TMAs from transgenic plants (0.02–0.05 g kg^{-1}) the cost of product purification significantly increases. To date, P-TMA costs produced in stably transformed plants are not competitive as they have reached €10,000–20,000 g^{-1} (Buyel and Fischer 2012) compared with ~€200 g^{-1} for CHO cells-derived TMAs (Nandi et al. 2016). Therefore, to make the plant production platform economically profitable, it is necessary to improve it by increasing the yield of P-TMAs to at least 2.0 mg kg^{-1}. In this way, it

is possible to use transient expression system, optimized VFUs, genetically engineering techniques (Zischewski et al. 2016) and techniques involving heat shock and the use of alpha lipoic acid in the agroinoculation mixture (Norkunas et al. 2018). These modification allow the costs for P-TMAs to fall substantially below €100 g^{-1} (Buyel and Fischer 2012).

24.9 Conclusions

1. Plants are promising factories for antibodies, as their cells have similar mechanisms to animal cells for protein synthesis and post-translational modification (glycosylation). P-TMAs have attracted biotechnologists due to their flexibility, speed, scalability, low production costs, and the absence of a risk of contamination by pathogens of animal origin.
2. Like most immunoglobulins in a healthy person, P-TMAs undergo glycosylation in the ER and Golgi apparatus in plant cells. The P-TMA Asn297-linked glycan is mainly of the complex type GnGnXF with a small number of MMXF type oligomannose glycans. The core portion of Asn297-glycan types GnGnXF and MMXF in P-TMAs contain 1,3-fucose and β1,2-xylose residues, which, in addition to the absence of a terminal galactose and sialic acid, distinguishes P-TMAs from human IgG.
3. The P-TMA Asn297-linked glycan is a specific plant glyco-epitope that is absent in mammals. P-TMA biosafety assessments in patient tests have indicated no side effects, although there is a fear that some plant glycans may be immunogenic to humans, and this has not been completely dispelled.
4. Different approaches to create P-TMAs that are maximally similar to human IgG in carbohydrate profile have been developed. The "humanisation" of the producer plant has the following aims: (1) to eliminate the immunogenicity of P-TMA, (2) the introduction of human genes into plant cells and the ability to synthesise P-TMA with the carbohydrate profile of human IgG, and (3) the suppression of β1,2-xylosylation and α1,3-fucosylation at the core portion of Asn297-linked glycans.
5. Several P-TMAs are at various stages of development but are still not approved for the pharmaceutical market. The best prospects of clinical use are for (a) P-TMAs aimed at treating viral (human immunodeficiency virus, Ebola virus, Junin virus, respiratory syncytial virus and flaviviruses) and bacterial (anthrax and botulism) infections and (b) anticancer P-TMAs.
6. To make plant products economically profitable, it is necessary to increase the yield of P-TMAs to at least 2.0 mg kg^{-1} which could be reached by using transient expression system, optimized VFUs and genetically engineering techniques.

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Droplet Digital PCR for Absolute Quantification of Plant Pathogens

25

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Abstract

Droplet digital PCR is the recent technological innovation that can detect up to a single gene copy. It features ease of use, precision, specificity and reproducibility for absolute quantification and gene expression studies than other ddPCR formats. ddPCR is a direct method to determine absolute quantification of target genes without the need of external standards. It is independent of amplification efficiency bias observed with quantitative PCR. ddPCR amplification of a sample is carried out in thousands of partitioned water-oil emulsion droplets and presence or absence of the target in each droplet is determined digitally by fluorescence at the end-point of amplification. Here, we discuss ddPCR applications of plant pathogens using citrus pathogens in duplex and triplex assays.

Keywords

ddPCR · Citrus · *Spiroplasma citri* · *Citrus tristeza virus* · *Hop stunt viroid*

25.1 Introduction

Plant pathogens pose a great threat to agriculture. Risks of pathogen spread have increased with increased human mobility and trade globalization. Agricultural exports offer economic opportunities but, at the same time, increase risk of exotic

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pathogen introduction to new areas. Early pathogen diagnosis can decrease this risk. Quantitative Real-Time Polymerase Chain Reaction (qPCR) enables precise and sensitive quantification of nucleic acids using fluorescent probe-based detection of nucleic acids and has become the gold standard for sensitive plant pathogen detection. Sykes et al. 1992 showed that absolute quantification of nucleic acid concentration could be done by combination of limiting dilution, end-point PCR, and Poisson statistics. Subsequently, the term "digital PCR" was coined and a method was developed to partition a sample to the extent that single template molecule can be amplified individually and resulting products can be detected using fluorescent probes (Vogelstein and Kinzler 1999). The resultant dPCR, now considered the third-generation PCR, involves partitioning the sample prior to amplification into thousands of partitions; each partition acts as an individual PCR reaction and contains either zero or few template molecules. Partitions containing an amplified fluorescent product are considered positive; whereas, those without fluorescence are considered negative. Digital PCR relies on the ratio of positives to negatives partitions in each sample and Poisson statistics is used to determine infection status. This increased precision and sensitivity allows dPCR to be considered as an early pathogen detection technology.

Digital PCR is now commercially available in different formats depending on strategies to produce partitions (droplets or chambers). Currently available dPCR platforms are BioMark HD (Fluidigm, South San Francisco, CA, USA), Clarity (JN Medsys, Singapore), and Quant Studio 12 flex and 3D instruments (Thermo Fisher, Waltham, MA) that partition samples into individual reaction wells on chips; whereas Rain Drop (Lexington, MA, USA) and QX100 and QX200 instruments (Bio-Rad, Hercules, CA, USA) partition samples using water-in-oil droplets which is called droplet digital PCR (ddPCR). In this chapter we discuss ddPCR and its application for detection of plant pathogens.

25.2 Droplet Digital PCR

Droplet Digital PCR has the advantages over other dPCR platforms by massive partitioning of the fluid phase of the sample without the need of complex fluidics used in chip-based system. ddPCR uses a water-oil emulsion droplet technology combining microfluidics and proprietary surfactant chemistries to divide PCR samples into thousands of nanoliter-sized water-in-oil droplets. Each droplet acts as an individual PCR reaction (Hindson et al. 2011). Sample partitioning is the key aspect in ddPCR.

Droplet digital PCR system is optimized for EvaGreen-based assay and TaqMan hydrolysis probe. EvaGreendouble-stranded (ds) DNA binding dye-based assay utilizes proprietary QX 200 ddPCR EvaGreen super mix and target-specific primers to quantify DNA pathogens. TaqMan hydrolysis probe assay utilizes target-specific primers, fluorescent probes (FAM and HEX or VIC) and ddPCR super mix for probes or 1-step RT-ddPCR kit to quantify nucleic acids (DNA or RNA) targets. The Bio-Rad system fractionates each PCR sample well (~20 μl) into 20,000 uniform

nanoliter-sized droplets. Droplets from each sample are transferred to a 96-well plate and sealed using the PX1 PCR Plate sealer. Each partitioned sample is then subjected to PCR amplification in a compatible thermal cycler, and each droplet is treated as an individual sample. After PCR, each droplet is analyzed by a two-color detection system (FAM and HEX or VIC) using a flow cytometric-based droplet reader. The instrument's Quantasoft™ software measures the fraction of positive and negative droplets for each fluorophore in the sample. The absolute copy number is determined by fitting the fraction of positive droplets in a Poisson distribution and results expressed as copies per microliter. The ddPCR reaction results are analyzed using the QuantaSoft Analysis Pro software.

25.3 Advantages of ddPCR over qPCR

Droplet digital PCR is gaining popularity by offering several advantages over conventional qPCR. It is a highly precise method for sensitive nucleic acid detection and absolute quantification. Sample partitioning prior to amplification and acquisition of end-point data makes ddPCR unique and versatile. In contrast, qPCR involves indirect method of quantification of pathogen genes using external standards of known quantity. The standard curve-based absolute quantification depends on PCR amplification efficiency. Generation of standards is complex and may deteriorate over time, thus, the initial quantity of the unknown sample may also be changed. ddPCR doesn't require any standards for quantification of the unknown sample. It is a direct method for absolute quantification of nucleic acids and independent of PCR amplification efficiency. Droplet digital PCR is less susceptible than qPCR to substances that inhibit PCR reactions and can produce reproducible data in presence of inhibitors. In addition, it is more tolerant to sequence variations and different types of DNA templates (Sanders et al. 2011). For very low target concentration in the sample, ddPCR has increased precision over qPCR. Although ddPCR has less dynamic range compared to qPCR, it provides unparalleled sensitivity for fractional abundance and discriminates very small changes in expression of genes up to 10%. ddPCR can quantify rare target sequence mutations in complex template backgrounds.

25.4 Application of ddPCR

Droplet digital PCR utilizing TaqMan probe hydrolysis probes and EvaGreen dsDNA binding dye has been used in different areas of biology for research and diagnostic purposes. Droplet digital PCR enables detection of rare mRNAs and miRNA in complex matrices such as blood (Ma et al. 2013). It was used to precisely quantify fine changes in gene expression of complex targets less than twofold levels (Zmienko et al. 2015). Droplet digital PCR allows accurate copy number variation quantification without the need of many replicates (Berman et al. 2012). Droplet digital PCR improved microbiome analysis due to high sensitivity, ability to amplify

rare targets in complex matrices and less prone to PCR inhibitors (Rački et al. 2014). ddPCR is a robust tool for single cell analysis for precise detection and amplification of low target templates without the need of standard curve or housekeeping genes. Droplet digital PCR is being used as reference tool for evaluation of different ddPCR and qPCR assays (Mehle et al. 2018). Droplet digital PCR multiplexing potential has been applied to accurately test genetically modified organisms and food contaminants (Morisset et al. 2013). It was also used for simultaneous detection of four targets through multiplexing and can be combined with other techniques such as next-generation sequencing (Dobnik et al. 2016, Aigrain et al. 2016). Droplet digital PCR has been increasingly used for absolute quantification and detection of plant pathogens such as fungi, bacteria, fastidious bacteria, viruses and viroids.

25.5 TaqMan Hydrolysis Probe-Based Quantification of Plant Pathogens

TaqMan probes are hydrolysis probes designed to anneal within a region amplified by specific primer sets and results in higher sensitivity, precision and absolute quantification. TaqMan probe-based ddPCR has been developed to quantify and detect the plant pathogens. The use of ddPCR for detection of plant pathogens was first developed by Dreo et al. 2014. The ddPCR was developed for routine detection and quantification of *Erwinina amylovora* and *Ralstonia solanacearum,* causal agent of fire blight of rosaceous plants and potato brown rot, respectively. Zhao et al. (2016) evaluated the diagnosis potential of ddPCR in comparison to qPCR for absolute quantification of *Xanthomonas citri* subsp *citri*, causal agent of citrus canker. Their results suggested that ddPCR assay has potential for absolute quantitative detection with high precision and accuracy compared to qPCR.

Hua et al. (2018) developed ddPCR assay for accurate quantification and differentiation of toxigenic and non-toxigenic strains of *Aspergillus flavus* from soil using *norA* and *omtA* genes respectively. *Cryphonectria parasitica* (Murill) Barr, is a latent pathogen that infects bark tissue and causes lethal bark cankers, wilting and dieback of chestnut trees. Chandelier et al. (2018) developed real time qPCR assay in relation to ddPCR to detect *C. parasitica* with a detection limit up to 2 fg of DNA equivalent to one copy of spore. Morella et al. (2018) developed ddPCR assay to enumerate two bacteriophages and its host bacteria, *Pseudomonas syringae,* to monitor its populations in vitro and in vivo, time-to-lysis and to explore phage-phage competition dynamics.

The first ddPCR assay for flavescencedorée, a phytoplasma-caused disease of grapevines targeted the *secY* gene (Nataša et al. 2014). Aster yellows, caused by *Candidatus* Phytoplasma asteris, is an important disease of brassicacious crops and is transmitted by the leafhopper, *Macrosteles quadrilineatus*. Bahar et al. (2018) developed a duplex ddPCR assay to detect this phytoplasma in infected plants using *16S* and *actin-2* gene primers and a singleplex assay targeting 16S rRNA in insect vector. "*Candidatus* Liberibacterasiaticus" (*C*Las) is a Gram-negative,

α-proteobacterium associated with Huanglongbing(HLB), a devastating citrus disease. Xi et al. (2018) developed ddPCR assay using positive plasmids with 16S gene specific primers and probes and found superior to qPCR for detecting *C*Las at low concentrations. Selvaraj et al. (2018) developed duplex ddPCR assay with RNR (ribonucleotide reductase) and 16S gene specific primers for *C*Las detection in comparison to qPCR. A duplex ddPCR reaction of both primers sets for detection of *C*Las in at very low concentrations showed promising results compared to singleplex reaction.

Lack of standards or reference materials has led to variability in quantification of plant viral RNA between experiments and laboratories. Mehle et al. (2018) developed a reverse transcriptase ddPCR assay for detection of *Potato virus Y* as a reference tool for evaluation of RT-qPCR assays. Specific detection of *Potato virus S* by ddPCR assay was developed by Zhang et al. (2017). The detection limit of PVS was 20 copies per µl of RNA, which was tenfold greater than RT-qPCR. Absolute quantification of plant virus in presence of inhibitory substances is a challenging in RT-qPCR. Rački et al. (2014) showed that, ddPCR has high resilience to inhibitory substances for quantification of Pepper mild mottle virus compared to RT-qPCR. Selvaraj et al. (2016) developed a triplex RT-ddPCR assay for detection of citrus pathogens viz., *S. citri*, *Citrus tristeza virus* and *Hop stunt viroid*.

25.6 EvaGreen Based Detection of Plant Pathogens

EvaGreen is a dsDNA binding dye designed based on release-on-demand mechanism. It is stable, non-toxic, non-mutagenic and environmentally safe dye, also involves reduced cost over TaqMan probe-based reactions. Currently, EvaGreen based ddPCR has been used to quantify bacteria and viruses in research and screening of germplasm. Voegel and Nelson (2018) developed a ddPCR assay to quantify *Agrobacterium vitis* causing crown gall disease in grapevine nursery stock and vineyard soil. Carmichael et al. (2018) quantified *Botrytis cinerea,* causal agent of gray mold, in asymptomatic table grapes and at different development stages of the berry. DeBlasio et al. (2018) used EvaGreen-based ddPCR to quantify the coat protein gene cDNA of *Potato leaf roll virus* (PLRV) and *Tobacco rattle virus* (TRV). Pinheiro et al. (2017) quantified titer of PLRV after 24 and 72 h of PLRV acquisitionin the aphid, *Myzuspersicae,* The absolute number of several plant RNA species, *Tomato bushy stunt virus*(*p19* ORF) and *Cucumber necrosis virus*(Coat protein and *p33* ORF) were quantified using ddPCR (Ghoshal et al. 2015). ddPCR was used to analyze the coat protein-mediated transgenic papaya lines and found that papaya line 474 contained a single copy of the transgene (Jia et al. 2017) (Table 25.1).

Table 25.1 Application of ddPCR for quantification of plant pathogens

Organism	Scientific name	Target Gene	Dye chemistry	Crop	References
Fungus	*Aspergillus flavus*	*omtA* and *norA*	TaqMan probes	soil	Hua et al. (2018)
	Cryphonectria parasitica	*ITS*	TaqMan	Chestnut	Chandelier et al. (2018)
Bacteria	*Pseudomonas syringae*	*16S*	TaqMan	Tomato	Morella et al. (2018)
	Bacteriophages	Coat Protein (CP)	TaqMan	Tomato	
	Agrobacterium vitis	*virA*	EvaGreen	Grapevine	Voegel and Nelson (2018)
	Erwinia amylovora and	*amsC*	TaqMan	Species of Rosaceae and Solanaceae	Dreo et al. (2014)
	Ralstonia solanacearum	*16S*	TaqMan		
	Xanthomonas citri Subsp. Citri	Hypothetical protein	TaqMan	Citrus	Zhao et al. (2016)
	Botrytis cinerea	*ITS*	EvaGreen	Grapevine	Carmichael et al. (2018)
Fastidious bacteria	Phytoplasma	unknown	TaqMan	Grapevine	Nataša et al. (2014)
	Candidatus Liberibacter asiaticus	*16S*	TaqMan	Citrus	Xi et al. (2018)
	Candidatus Phytoplasma asteris	*16S and actin*	TaqMan	Canola	Bahar et al. (2018)
	Candidatus Liberibacter asiaticus	*RNR, 16S*	TaqMan	Citrus	Selvaraj et al. (2018)
	Spiroplasma citri	*ORF, 16S*	TaqMan	Citrus	Maheshwari et al. (2017)
Virus	*Potato virus Y*	Coat protein	TaqMan	Potato	Mehle et al. (2018)
	Potato leaf roll virus	Coat protein	EvaGreen	*Nicotiana benthamiana* and *Solanum sarrachoides*	DeBlasio et al. (2018)
	Tobacco rattle virus	Coat protein	EvaGreen		
	Potato leaf roll virus	Coat protein	EvaGreen	*Vector: Myzus persicae*	Pinheiro et al. (2017)
	Cucumber necrosis virus	Coat protein and *P33*	EvaGreen	*Nicotiana benthamiana*	Ghoshal et al. (2015)
	Tomato bushy stunt virus	*P19*	EvaGreen	*Nicotiana benthamiana*	
	Papaya ringspot virus	Coat protein	EvaGreen	Papaya	Jia et al. (2017)
	Potato virus S	Coat protein	TaqMan	Potato	Zhang et al. (2017)
	Pepper mild mottle virus	Replicase	TaqMan	*Capsicum annuum*	Rački et al. (2014)
Viroid	Hop stunt viroid	Genome	TaqMan	citrus	Selvaraj et al. (2016)

25.7 Absolute Quantification of Citrus Pathogens

Symptoms induced by citrus pathogens can be confounded by co-infection of multiple pathogens, host susceptibility and environmental conditions. Problems for accurate quantification and detection includes, but is not limited to, competition between pathogen targets, erratic distribution and low titer of target pathogens. Since ddPCR provides highest precision, sensitivity and resilience to PCR inhibitors, we present a workflow for quantification of citrus pathogens by duplex assay (*C*Las and *Spiroplasma citri*) and triplex assay (*Citrus tristeza virus*(CTV), *Hop stunt viroid* (HSVd), and *S. citri*). The ddPCR system described here provided absolute quantification of target nucleic acids optimized for TaqMan hydrolysis probes (Table 25.2).

25.8 Duplex ddPCR Based Detection of *C*Las and *S. citri*

Target genes for *C*Las was RNR; and for *S. citri* was ORF 1. Total nucleic acids (TNA) was extracted from *C*Las- and *S. citri*-dually infected leaf samples by CTAB method. The ddPCR reaction mixture was prepared as described in Table 25.3. The reaction mixture was loaded into an eight well disposable plastic cartridge together with 70 µl of droplet generation oil and placed in the QX200 droplet generator. The entire droplet emulsion volume was further loaded in a semi-skirted and PCR-clean 96-well plate (Eppendorf, Leuven, Belgium). The plate was then heat sealed with pierceable foil in the PX1 PCR plate sealer and PCR amplification was carried out in a S1000 thermal cycler (Bio-Rad). The thermal cycling conditions consisted of 10 min enzyme activation at 95 °C, followed by 40 cycles of a two-step thermal profile of 30 s denaturation at 94 °C and one-minute annealing-extension at 60 °C with a ramp rate of 2 °C, and a final 10 min denaturation step at 98 °C. After thermal cycling, plates were transferred to the QX200 droplet reader (Bio-Rad, CA, USA). The samples were tested in three replicates. The ddPCR data were analyzed with QuantaSoft software version 1.7 (Bio-Rad). The reactions with more than 10,000 accepted droplets per well were used for analysis. The positive droplets containing amplified products were discriminated from negative droplets by applying a threshold of 4000 for FAM channel and 2000 for VIC channel. The copy number concentration of each sample was reported automatically by ddPCR software. The copy number in 20 µl reaction was detected as 7360 and 1390 for *C*Las and *S. citri* respectively, in the duplex ddPCR assay (Fig. 25.1).

25.9 Triplex One-Step RT-ddPCR Based Detection of *S. citri*, CTV and HSVd

Target genes for *S. citri* was ORF1;CTV was coat protein gene; HSVd was a conserved region of the genome. The total nucleic acids (TNA) was extracted from dually infected (*S. citri* and CTV) and HSVd-infected leaf samples by CTAB

Table 25.2 Primer and probes

Pathogen	Target gene	Primer/probe	Sequence	References
Candidatus Liberibacter asiaticus	RNR	RNR F	CATGCTCCATGAAGCTACCC	Zheng et al. (2016)
		RNR R	GGAGCATTTAACCCCACGAA	
		RNR probe	6FAM/CCTCGAAATCGCCTATGCAC/MGB/NFQ	
Spiroplasma citri	SpV1-ORF1	ORF 1-F	TGGCAGTTTTGTTTAGTCATCC	Wang et al. (2015)
	Prophage	ORF 1-R	GGGTCTAAACGCCGTTAAAGT	
		ORF 1 FAM	6FAM/TTGGGTTTGGTTATTCCATT/MGB/NFQ	Maheshwari et al. (2017)
		ORF 1 VIC	VIC/TTGGGTTTGGTTATTCCATT/MGB/NFQ	
Citrus tristeza virus	Coat Protein	P25F	AGCRGTTAAGAGTTCATCATTRC	Saponari et al. (2008)
		P25R	TCRGTCCAAAGTTTGTCAGACA	
		CP HEX	5HEX/CRCCACGGGYATAACGTACACTCGG/BHQ1	
Hop stunt viroid	Conserved genome	HSVd-F	CTCTTCTCAGAATCCAGCG	Saponari et al. (2013)
		HSVd-R	GGACGATCGATGGTGTTTCGAA	
		HSVd-FAM	AGAGAGGGCCGCGGTGCTC/MGB/NFQ	
		HSVd-HEX	5HEX/AGAGAGGGCCGCGGTGCTC/BHQ1	

Table 25.3 Reaction set up for ddPCR duplex assay

Component	Final concentration
Template	~50 ng
ddPCR Supermix for probes (No dUTP)	2X
Target primers[a]	900 nM
*C*Las FAM probe	250 nM
S. citri VIC probe	250 nM

[a]Forward and reverse primers of *C*Las and *S. citri*

Nuclease free water is used to make up a final volume of 20 µl

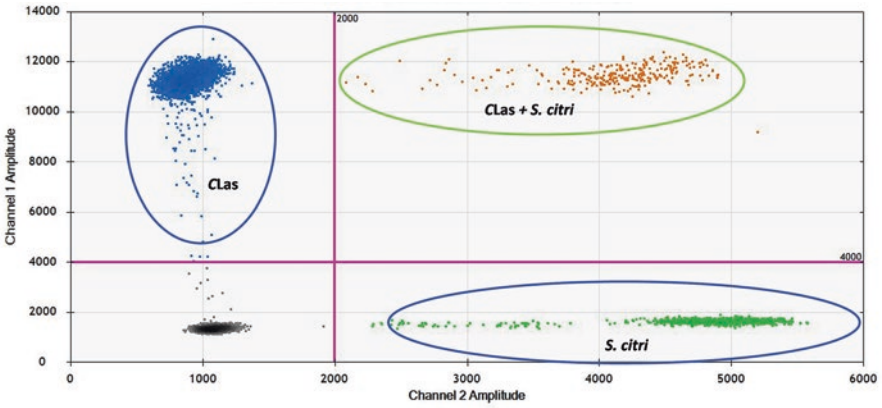

Fig. 25.1 Absolute quantification of *S. citri* and *C*Las by duplex ddPCR in infected leaf samples. Each distinct cluster of dots represents droplets that are positive for a single or multiple target. Blue circles showing single positive population and Green circle showing double positive population. Positive droplets containing amplified products were discriminated from negative droplets by applying a threshold of 4000 for FAM channel and 2500 for VIC channel. (The figure was obtained from QuantaSoft™ Analysis Pro 1.0.596)

method. Approximately 10–15 ng of TNA was mixed together and utilized for ddPCR assay. The RT-ddPCR reaction mixture was prepared as described in Table 25.4. The reaction mixture was loaded into a disposable plastic cartridge together with 70 µl of droplet generation oil and placed in the QX200 droplet generator. The entire droplet emulsion volume was further loaded in a semi-skirted and PCR-clean 96-well plate (Eppendorf, Leuven, Belgium). The plate was heat-sealed with pierceable foil by a PX1 PCR plate sealer and PCR amplification was carried out in a S1000 thermal cycler. The thermal cycling conditions consisted of 60 min reverse transcription at 60 °C, 10 min initial denaturation at 95 °C, followed by 40 cycles of a two-step thermal profile of 30 s denaturation at 95 °C and 60 s

Table 25.4 Reaction set up for RT-ddPCR triplex assay

Component	Final concentration
Template	~10–15 ng
Supermix	1X
Reverse transcriptase	20 U/µl
Dithiothreitol	15 mM
Target primers[a]	900 nM
S. citri ORF-1 FAM probe	250 nM
CTV CP HEX probe	250 nM
HSVd FAM probe	125 nM
HSVd HEX probe	125 nM

[a]Forward and reverse primers of *S. citri*, CTV and HSVd

Nuclease free water is used to make the final volume 20 µl

annealing-elongation at 57 °C with a ramp rate of 2 °C, and a final 10 min denaturation step at 98 °C. After thermal cycling, plates were transferred to the QX 200 droplet reader (Bio-Rad, CA, USA). The samples were tested in three replicates. The ddPCR data were analyzed with QuantaSoft software version 1.7 (Bio-Rad). The positive droplets containing amplified products were discriminated from negative droplets by applying a threshold of 5000 for FAM channel and 1500 for HEX channel. The reactions with more than 10,000 accepted droplets per well were used for analysis. The copy number concentration of each sample was reported automatically by ddPCR software. The copy number in 20 µl reaction was detected as 85, 8150, 4272 respectively, for *S. citri,* CTV and HSVd, respectively, in the triplex ddPCR assay (Fig. 25.2).

25.10 Conclusion

Droplet Digital PCR fractionates a sample for digital PCR into 20,000 water-oil emulsion droplets and PCR amplification is performed on each droplet. Droplet Digital PCR uses reagents and workflows like those used for most standard TaqMan probe-based assays. The massive sample partitioning and the use of Poisson statistics to determine the absolute copy number of the target molecules present without need of reference standards is a key advantage of the method. Droplet partitioning of the sample also dilutes PCR inhibitors and non-target DNA resulting in great tolerance to inhibitors and an increase in ratio of target to

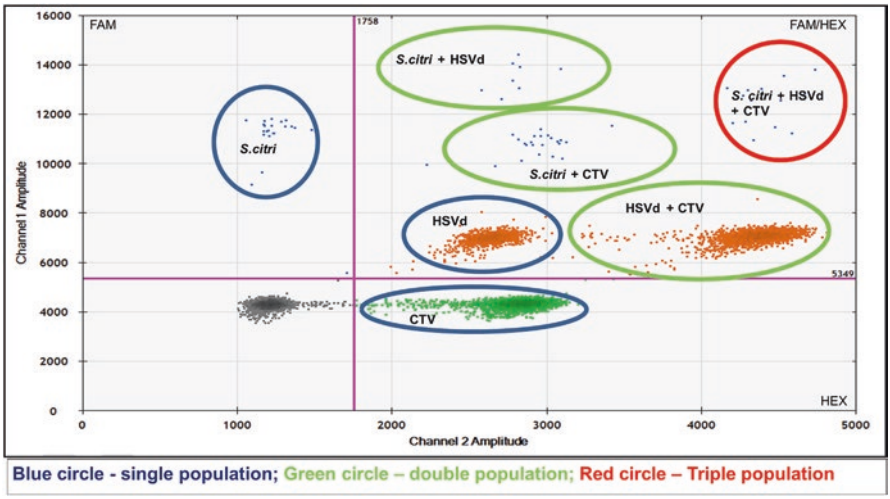

Fig. 25.2 Absolute quantification of citrus pathogens by triplex RT-ddPCR. Each distinct cluster of dots represents droplets that are positive for a single or multiple target. Blue circles showing single positive population, Green circle showing double positive population and red circle is triple positive population. Positive droplets containing amplified products were discriminated from negative droplets by applying a threshold of 5000 for FAM channel and 1500 for HEX channel. (The figure was obtained from QuantaSoft™ Analysis Pro 1.0.596)

primer/probe per droplet. The end result is a highly robust and sensitive amplification of a target DNA or gene with a direct measure of the absolute copy number of the target molecule.

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Diagnostics for Citrus Greening Disease (Huanglongbing): Current and Emerging Technologies

26

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Abstract

Huanglongbing (HLB) or 'citrus greening' is the most destructive disease in citrus production worldwide and no cure is available at present. This disease is caused by a phloem-limited, non-cultured, Gram-negative alpha-proteobacteria, *Candidatus* Liberibacter spp., vectored by the psyllids, *Diaphorina citri* and *Trioza erytreae*. Practically all commercial citrus species and cultivars are sensitive, regardless of rootstocks used. Foliar symptoms produced by HLB can easily go unnoticed and often be confused with those induced by nutrient deficiencies or other endemic diseases. Low concentration and the uneven spatial and temporal distribution of the pathogen in host plants and insect vectors make the detection even more difficult. Currently molecular techniques based on polymerase chain reaction are mainly used for the identification of HLB disease, which requires exhaustive sampling and processing operations. Furthermore, researches are ongoing in spectroscopic and imaging techniques, profiling of plant volatile organic compounds, and isothermal amplification approaches. This review provides a comprehensive overview on the various diagnostic techniques available for HLB and also discusses the recent advancements in HLB disease detection.

Keywords

Huanglongbing · HLB · PCR · Diagnosis · VOC

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26.1 Introduction

'Citrus greening' (aka Huanglongbing, abbreviated as HLB, a Chinese name meaning yellow shoot disease) is a devastating bacterial disease causing severe losses for the citrus industry worldwide (Khan and Razi 2018). It affects all citrus cultivars and causes decline of trees. The disease has seriously impacted citrus production in most citrus-producing regions of the world (da Graça et al. 2016) and currently distributed in varied countries and regions of Asia, Africa, South and North America (Haapalainen 2014). It is spread by vegetative propagation and by insect vectors (Bové 2006), two reasons why HLB has become such a problematic disease to control. The identification of HLB infection is highly challenging because of its similarity to the symptoms of such nutrient deficiencies of Zn and Fe, and its putative causal agent cannot be cultivated on an artificial culture medium (Wang et al. 2017). In addition, no effective agrochemicals are yet available to control this disease. The only counter measure to cope up with HLB infection is to cut down the infected citrus trees, replacing them with disease-free nursery plants and control of insect vectors (Gottwald et al. 2007). Therefore, early detection is very important to mitigate the damage caused by HLB infection. HLB is associated with species of phloem-limited Gram-negative bacteria, named '*Candidatus* Liberibacter' (Jagoueix et al. 1994), after the Latin word *liber* meaning 'bark'. The term *Candidatus* indicates that it has not yet been possible to maintain this bacterium in culture. At present, there are three species of the genus '*Candidatus* Liberibacter' identified to be responsible for causing HLB disease (based on reinfection studies even though Koch's postulates cannot be fulfilled) in citrus plants are: a heat-tolerant bacterial species '*Ca*. Liberibacter asiaticus' (CLas), vectored by the Asian citrus psyllid (ACP), *Diaphorina citri* (Capoor et al. 1967; Martinez and Wallace 1967) in Asia, Brazil, and Florida (USA) (Halbert 2005); a heat-sensitive species '*Ca*. Liberibacter africanus' (CLaf) transmitted by the African citrus psyllid *Trioza erytreae* in the African countries (Aubert 1987; Garnier and Bovè 1993; Bové 2006); and the heat-sensitive species '*Ca.* Liberibacter americanus' (CLam) transmitted by *Diaphorina citri* in Brazil (Texeira et al. 2005a, b; Lopes et al. 2009). Though phytoplasmas belonging to several 16Sr groups have been reported to be associated with HLB symptoms (Teixeira et al. 2008; Lou et al. 2014; Das et al. 2016; Wulff et al. 2019), it's foolproof involvement as a disease causing agent has not been established so far. Mottling and yellowing of leaves, lopsided, small, premature fruit and excessive fruit drop are the variable symptoms associated with HLB. Currently HLB has established itself in more than 40 countries throughout the world (Iftikhar et al. 2016) and in the areas affected by the disease, the average productive lifespan of citrus trees has dropped from 50 or more years to 15 or less (USDA-APHIS 2016). In view of the necessity and seriousness of this problem, researchers all over the world are working towards the development of a rapid, early, less-costly and accurate detection system for strengthening HLB management strategies and quite a few reviews have been published of late (Li and Levy 2009; Iftikhar et al. 2016; Valdes et al. 2016). This review discusses the emerging and upcoming technologies in addition to traditional and current methods available to diagnose HLB.

26.2 Origin and Distribution of Huanglongbing (HLB)

The origins of HLB are still a matter of debate. The initial record of HLB disease was from China, dates back to 1919 in Chaozhou district of Guangdong province (Reinking 1919). While early reviewers cited observations in southern China as the center of origin of the Asian form of the disease (Zhao 1981; da Graça 1991; Bové 2006), reports and data from the Indian subcontinent (Husain and Nath 1927; Capoor 1963) had been overlooked, and there is a possibility that this is where citrus was first infected by CLas, with subsequent movement of infected plant material to other Asian countries (Beattie et al. 2008). The African form of the pathogen, CLaf probably originated from native African Rutaceae family, and several have been found to be infected with variants of CLaf (Roberts et al. 2015). The initial confirmation of HLB in Brazil was associated with a previously unknown species, CLam, but it is being displaced by CLas probably because the latter is more heat tolerant and reaches higher titers in citrus (Gasparoto et al. 2012). It is suggested that it may be indigenous to the Americas (da Graca et al. 2015).

Throughout last century HLB disease has prevailed and affected citrus groves in China, Taiwan, India, Sri Lanka, Malaysia, Indonesia, Myanmar, the Philippines, Pakistan, Thailand, the Ryukyu Islands of Japan, Reunion, Nepal, Saudi Arabia, and Afghanistan in Asia and South Africa and Zimbabwe in Africa (Garnier and Bove 1996; Halbert and Manjunath 2004). In 1996 almost 100% of mandarin trees in northern Bali were severely affected with HLB (Bove 2006). Fraser et al. (1966) reported occurrence of HLB in India for the first time. Prevalence of HLB was later reported from different parts of India (Raychaudhuri et al. 1971; Gupta et al. 1972; Ahlawat and Sardar 1976; Naidu and Govindu 1981; Nariani 1981; Varma et al. 1993; Das and Singh 1999; Das et al. 2014). In the year 2004, it was reported for the first time in western hemisphere of the world (Sao Paulo state of Brazil). Thereafter HLB has moved into neighbouring states in Brazil as well as Argentina and Paraguay (Lopes et al. 2013). The presence of Asian citrus psyllid (ACP) was reported in Florida, USA in 1998 (Tsai and Liu 2000), but the subsequent HLB infection in citrus plants was reported in 2005 (https://crec.ifas.ufl.edu/extension/greening/history.shtml), which is almost after 6 years of ACP invasion.

In the USA, HLB has been detected in two other significant citrus producing states, Texas (Kunta et al. 2012; da Graca et al. 2015) and California (Kumagai et al. 2013) as well as in South Carolina, Georgia and Louisiana (Halbert et al. 2010). The disease is also widespread in several central and South American countries such as Cuba (Luis et al. 2009), Jamaica (Oberheim et al. 2011), Belize (Manjunath et al. 2010), Argentina (Badaracco et al. 2017) and Mexico (Trujillo-Arriga et al. 2010). Other major citrus growing parts of the world like Australia and the Mediterranean Basin are now under threat. HLB has moved west from Pakistan into Iran (Faghihi et al. 2009), threatening Turkey and beyond, and the African psyllid was recently found in Spain and Portugal (Cocuzza et al. 2017). HLB was detected in Papua New Guinea in 2002 (Weinert et al. 2004) causing Australia to step up its biosecurity measures. Recently first occurrence of *D. citri* was recorded in Tanzania, East Africa (Shimwela et al. 2016).

26.3 Economic Loss

Citrus is the most extensively produced tree fruit crop in the world and in terms of international trade, citrus fruit is one of the most imperative produce and occupies top position in international trade for its economic significance (Liu et al. 2012). At present HLB is the prime threat to the worldwide sustainable and commercial citrus production. It is destructive (probably is the most serious disease of commercial sweet orange and mandarin cultivars) irrespective of different rootstock combinations used or whether the trees are budded or are seedling trees (Gottwald 2010). The yield of affected trees is not only reduced considerably by persistent fruit drop, dieback, and tree stunting, but also by the inferior quality of fruits that remain on the trees (Batool et al. 2007).

HLB epidemics take many years to reach high incidence levels. The progress of disease is dependent on (i) insect vector populations, (ii) extent of the pathogenic inoculum reservoir, and (iii) age of the orchard at first infection. Severe symptoms have been observed 1–5 years after onset of the first symptoms, depending on the age of the tree at infection time and on the intensity of infection (Aubert 1992). As the disease severity increases, the yield declines and orchard production becomes uneconomical (Gottwald et al. 1991; Roistacher 1996). It is estimated that close to 100 million citrus trees are affected by HLB worldwide (National Research Council 2010). It takes 2–5 years for a tree to become unproductive from the first appearance of the symptoms and the total life span of the tree is reduced to 7–10 years, although significant quantitative data on fruit yield and quality reduction due to this disease are absent (Iftikhar et al. 2016).

In early 1900s, over 60 million trees were declined globally (Aubert 1993). Single-handedly HLB was responsible for the devastation of three million trees in Indonesia (Tirtawadja 1980). HLB has been a particularly devastating disease problem in Florida. In 2005, HLB was first reported in Florida region of United States and according to the survey conducted in 2009, nearly 60,000 acres of citrus was destroyed by HLB. In Florida alone, HLB has estimated to cause a cumulative loss of 2.994 billion US dollar in growers' revenues over the period of 2006–07 to 2013–14, an average loss of 374 million US dollar/year and also led to the loss of 6600 jobs (National Academies of Sciences, Engineering and Medicine 2018). As compared to the other pathosystems, HLB have rapid spatial spread and devastating economic impact. In Africa nearly, 30–100% of decrease in citrus yield is due to HLB (da Graca and Korsten 2004). A survey conducted over an 8-year period in Réunion Island indicated that 65% of the trees were badly damaged and rendered unproductive within 7 years after planting (Aubert 1992). In Thailand, citrus trees declined within 5–8 years after planting due to HLB (Roistacher 1996). Infected trees are stunted and sparsely foliated. In India, based on the field observation and information provided by the growers, HLB causes heavy loss to citrus orchardists. For example if 100 trees in an orchard with 1000 trees are declined within 5th year, the total loss to the grower would be ₹ 1.16 lakhs (Ahlawat and Pant 2004). This indicates the enormity of the problem in Indian subcontinent as thousands of trees were reported to have declined due to HLB (Das and Singh 1999; Ghosh et al. 2003; Das et al. 2002).

26.4 Taxonomic Information of the Causal Agent

The presumed causal organisms of HLB were first assigned to the genus, '*Candidatus* Liberobacter' (Jagoueix et al. 1994). However, 'Liberobacter' is orthographically incorrect and the generic name was subsequently amended to 'Liberibacter' (Jagoueix et al. 1997). The term *Candidatus* is used with this genus to signify that the organism has been partially genetically characterised and that other characteristics, required by the International Code of Nomenclature of Bacteria to determine its exact phylogenetic position, are lacking (Murray and Schleifer 1994).

The taxonomic hierarchy of the Liberibacters is given below:

Kingdom: Bacteria
Phylum: Proteobacteria
Class: Alphaproteobacteria
Order: Rhizobiales
Family: Rhizobiaceae
Genus: Liberibacter

Within the genus, three species infecting Rutaceae and four subspecies have been recognised:

'*Candidatus* Liberibacter asiaticus' Jagoueix et al. (1994)
'*Candidatus* Liberibacter africanus' Jagoueix et al. (1994)
'*Candidatus* Liberibacter americanus' Teixeira et al. (2005b)

Four additional subspecies of CLaf have also been recognized: *Candidatus* Liberibacter africanus subsp. capensis (CLafC), *Candidatus* Liberibacter africanus subsp. clausenae (CLafCl), *Candidatus* Liberibacter africanus subsp. zanthoxyli (CLafZ) and *Candidatus* Liberibacter africanus subsp. vepridis (CLafV) (Garnier et al. 2000; Roberts et al. 2015).

Worldwide distribution map of different *Ca*. Liberibacter spp. is shown in Fig. 26.1.

26.5 Detection of Huanglongbing

26.5.1 Visual Symptoms and Biodiagnosis

HLB symptoms are variable and can resemble other disorders of citrus. Symptoms usually diverge according to variety/cultivar, tree maturity, time of infection, stage of disease, and other abiotic factors or biotic agents that affect the tree. All citrus seedling trees and scion-rootstock combinations are sensitive to HLB (Ahlawat 1997; Bove 2006).

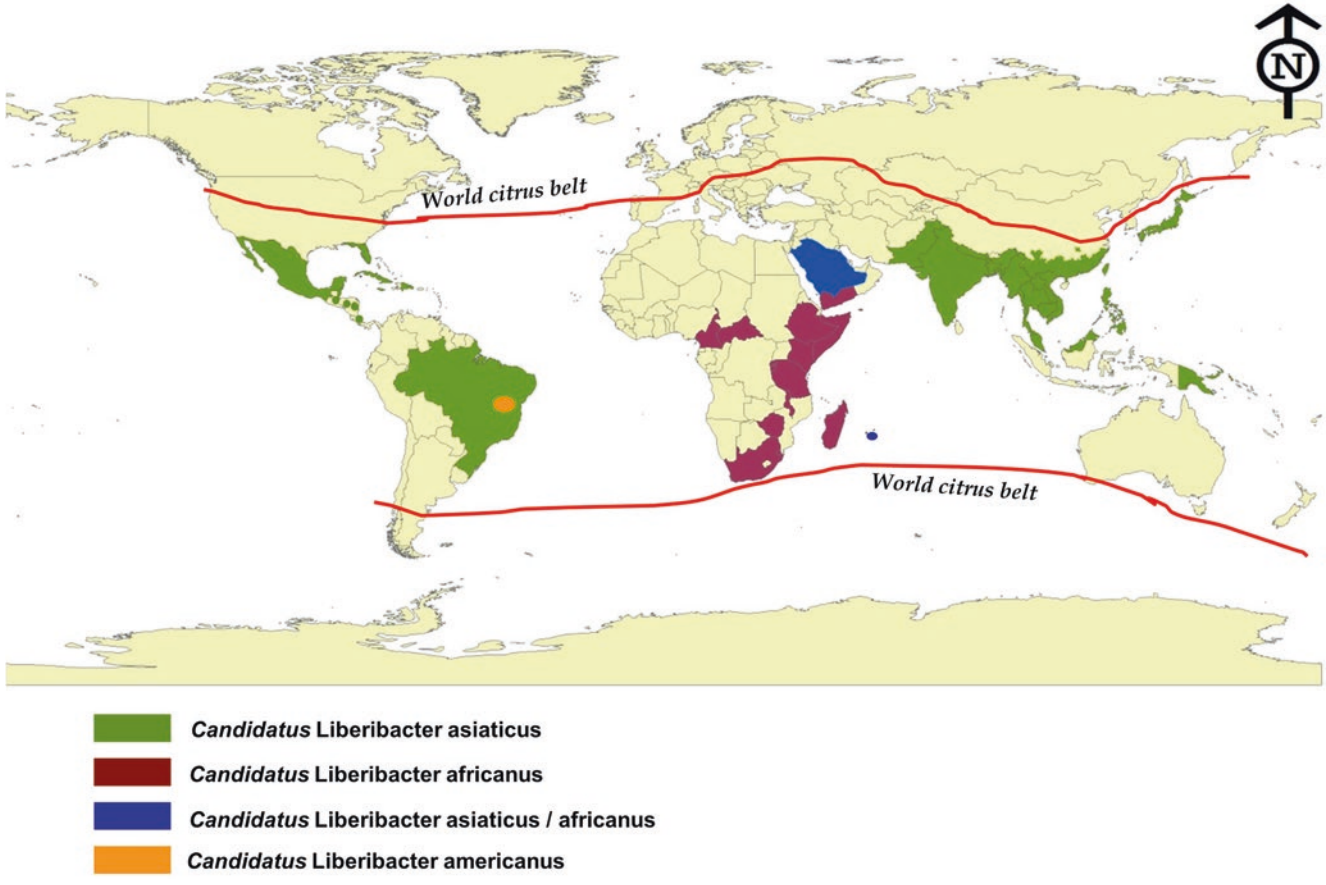

Fig. 26.1 Global distribution of citrus greening disease (Huanglongbing)

26.5.1.1 Tree Symptoms

The first indication of the disease is usually the appearance of a yellow shoot on a tree, hence the name 'huang-long-bing' which literally means yellow shoot (or yellow dragon as per Chinese mythology) disease. As the disease advances, twigs begin to display a greater degree of yellowing, sometimes accompanied by erect growth that may show "rabbit eared" leaves (Gómez 2008). After sometime, twigs begin to die and the tree begins to decline quickly towards its eventual death. In some cases, the progression of the symptoms results in an excessive drop of leaves and fruits. At the early stages of infection, infested trees are identified among symptomless trees by the presence of one or several characteristic "yellow shoots" (Fig. 26.2a). Afterwards, the yellow shoot develops into a larger yellow or pale green branch. Yellow branches may show one or several of the following features: defoliation, twig dieback, fruit drop, and presence of leaves with blotchy mottle and nutrient deficiency-like symptoms (Gottwald et al. 2007).

26.5.1.2 Symptoms on Leaves

The most important characteristic symptom of HLB, worldwide, is leaf "blotchy" mottle (primary symptom) (Mcclean and Schwarz 1970). Among the commercial citrus varieties, the sweet orange leaves (Fig. 26.2b) show classic "blotchy" mottle symptom very promonently, characteristic of HLB. This mottled leaf has several shades of yellow, pale green and dark green. These shades intermingle into each other, and there are no sharp borders between the various shades of colour, hence the term "blotchy" mottle (Bove 2006). This asymmetry helps to distinguish blotchy mottle from deficiency symptoms due, in particular, to lack of zinc, manganese or magnesium. Asymmetric blotchy leaf mottle (Fig. 26.2c–e) is the most diagnostic symptom of HLB in different citrus germplasm and varieties (Gómez 2008). The mottling normally crosses leaf veins, but not crosses the mid vein. With trees in early stages of disease, blotchy mottle may affect the well-developed leaves, and may be the only leaf pattern to be seen. In later stages, foliar symptoms of zinc deficiency (secondary symptoms) will eventually develop, such leaves will remain small, and thereafter, the whole leaf blade may ultimately turn uniformly yellow. Other symptoms include yellowing of the leaf veins which can become enlarged, swollen, and corky especially on the upper side of the leaf.

26.5.1.3 Symptoms on Fruit

In addition to blotchy mottle on leaves, HLB also exhibit characteristic symptoms on fruit. On normal fruit, when fruit ripens and changes colour, the orange colour develops first at the stylar (lower) end, at a time when the peduncular (upper) end is still green. On HLB-affected fruit, there is colour inversion: the orange colour starts first at the peduncular (upper) end, at a time when the stylar (lower) end is still green (Fig. 26.3). Colour inversion is particularly pronounced in cooler regions of the world viz. China, where it is called "red nose". This very symptom was responsible for calling the disease "greening" in South Africa (Bove 2006). Many of such HLB-infected Nagpur mandarin fruits showing stylar end greening symptoms are called *Wai-var* (Marathi language meaning 'a waste' or 'useless') in the Paratwada-Achalpur

Fig. 26.2 Various symptoms associated with Huanglongbing. (**a**) Emergence of yellow shoot from the upper canopy of a Mosambi sweet orange tree. Classic asymmetric blotchy mottle symptoms due to HLB infection in leaves of (**b**) Mosambi sweet orange, (**c**) Lisbon lemon, (**d**) Gol nimboo (*C. jambhiri*) and (**e**) Assam lemon

region of Amravati district, Maharashtra state, India. Cross-sections through the fruit axis are especially useful to observe not only the orange-stained vascular bundles in the peduncular half of the fruit axis, but also the presence of aborted seeds, and the asymmetry or lopsidedness of the fruit. The central axis of the fruit (in vertical cross section) is curved in response to the fruit's misshapenness, Lopsided fruits are characteristic of HLB, and their size is smaller than that of normal fruits. Reduced fruit size is one of the symptoms produced by HLB that is evident in

Fig. 26.3 Fruit symptoms of citrus greening in Nagpur mandarin (above) and Kinnow mandarin (below)

orchard and is responsible for losses in the fresh fruit market and the juice from fruits affected by HLB has been bitter and with a low content of soluble acids, or as having a salty bitter taste (Gómez 2008).

26.5.1.4 Root Loss

Johnson et al. (2014) found that HLB causes a loss of 30–50% of trees' fibrous roots before symptoms are visible above ground. HLB bacteria enter trees through leaves but the disease attacks roots long before the leaves show signs of damage. Once bacteria enters the plant system when psyllid sucks into leaves, the bacteria travel rapidly to the roots, where they replicate, damage the root system and spread to the rest of the host tree's canopy (Johnson et al. 2014).

26.5.1.5 Biological Indexing

Although PCR-based techniques are currently the method of choice for diagnosis of HLB *in planta*, biological indexing using indicator plants is the most easy and cheapest way to detect HLB infection. In this technique, seedlings of indicator plants are inoculated with side garfts/buds of a given source (or suspected) tree

(Fig. 26.4). The inoculated plants are kept in screen house for symptom development under controlled temperature conditions. Recommended indicator plants are seedlings of sweet orange (Mosambi, Malta, Valencia, Hamlin etc.) or, mandarin (Ponkan, Darjeeling, Coorg etc.) varieties (Das et al. 2002). Typical symptoms of disease generally appear with the first emerging shoots within 3–4 months after inoculation. Symptom expression is the typical mottle and chlorosis. The shoots are distinctly smaller, more chlorotic, and with smaller leaves than the uninoculated controls. Due to frequent low rate of graft transmission of the bacterium associated with HLB, the success rate for biological indexing is variable. Presence of the citrus tristeza virus (CTV) can interfere with HLB symptom expression and if CTV is present, grapefruit or trifoliate orange may be used as an indicator host (Roistacher 1991).

26.5.2 Microscopic and Biochemical Techniques

Formerly, The electron microscopy (EM) was the only reliable detection method for the HLB bacterium. The first EM observation of a "mycoplasma-like organism" in phloem tissues of HLB-infected citrus was carried out in 1970 (Lafleche and Bove 1970). Afterwards examinations of the disease-affected tissues, collected from South Africa ("greening"), India ("dieback"), Taiwan ("likubin") and Philippines ("mottle leaf"), using transmission electron microscopy (TEM) confirmed that the causative agent of HLB as a bacterium and also revealed that the pathogenic bacterium possesses a cell wall of the Gram-negative type and exclusively resides within the sieve tubes of infected citrus trees (Garnier and Bove 1983; Garnier et al. 1984; Naidu and Govindu 1981). This microscopic technique proved very reliable for a number of years prior to the development of detection methods based on DNA hybridization and PCR. Later Folimonova and Achor (2010) also conducted TEM,

Fig. 26.4 Biological indexing, though traditional, still remains an important technique to detect and maintain HLB bacterium in citrus hosts. Expression of blotchy mottle symptom on Mosambi sweet orange indicator seedling (right)

on leaves, petioles and stem tissues to detect the bacteria from HLB affected 'Madam Vinous' sweet orange trees. Tanaka et al. (2007) detected CLam in infected phloem vessels of *Catharantus roseus* (experimentally infected by CLam using dodder), using scanning electron microscopy (SEM) in Brazil.

In a light microscope study of symptom development, Schneider (1968) reported the build-up of large amounts of starch in leaves of HLB affected trees. HLB can be detected by an iodine test for starch accumulation after leaf symptoms are visible. The underside of the test leaf can be scratched with cloth-backed sandpaper and the sand paper placed in the iodine solution (Fig. 26.5), or the cut edge of the leaf can be submerged in the iodine solution for 30 s to 1 min to produce the black iodine-starch complex (Takushi et al. 2007; Etxeberria et al. 2007). In fact, due to its simplicity, reliability, rapidity, and cost efficiency, iodine-based starch tests have been used as visual field tests for HLB presence in many Asian countries (Taba et al. 2006; Takushi et al. 2007; Adhikari et al. 2012; Nurwahyuni et al. 2015) and Florida, USA (Etxeberria et al. 2007).

Another method based on the identification of a fluorescent gentiosyl-glucoside from infected fruits and bark tissues using fluorescent chromatographic technique was developed in 1968 in South Africa (Schwarz 1968) for confirmation of the disease. The method has since been widely used in India for rapid detection of HLB (Gupta et al. 1972; Dakshinamurti et al. 1976; Cheema and Kapur 1981) However, this method soon proved non-specific since stressed trees contained the same marker and hence it cannot be used alone for definitive diagnosis (Hooker et al. 1993).

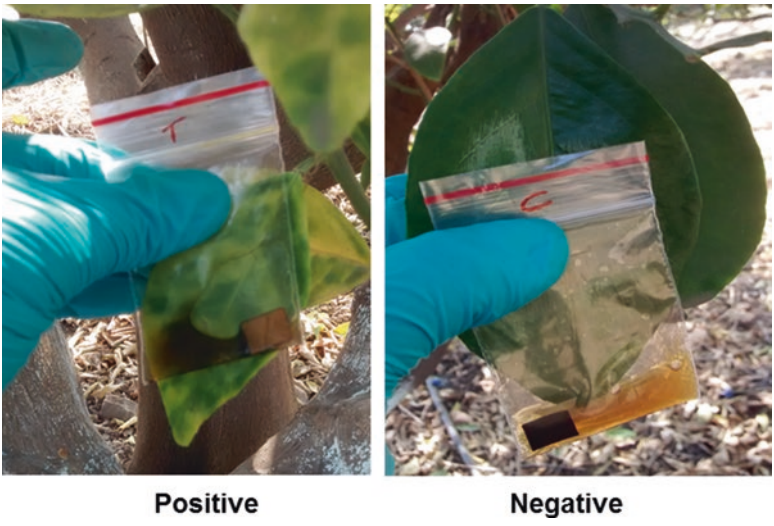

Fig. 26.5 Iodine solution starch test using sand paper to abrade leaf surface and then sandpaper is dipped in iodine solution. Note dark solution in left bag from starch in HLB positive sample

26.5.3 Serological and DNA Hybridization Assay

Antibodies have long been a cornerstone in the study of plant pathogens, and are applied to detect, identify and quantify pathogens. Because CLas has not been cultured, conventional polyclonal antibodies (PAbs) raised against the whole bacterial cell have not been produced. However the genome of CLas has been determined (Duan et al. 2009), which makes proteins of CLas available for use as antigens by PCR-based cloning. Ding et al. (2016) reported that the combination of a rabbit polyclonal antiserum against the major outer membrane protein (*omp*) of CLas and a secondary goat anti-rabbit PAb provided specific detection of CLas in the direct tissue blot immunoassay (DTBIA). *Omp* protein of CLas was also used earlier for development of PAbs (Lu et al. 2013). Two peptides containing hydrophobic polypeptide-transport-associated (POTRA) domain and β-barrel domain were identified and in vitro over expressed in *Escherichia coli*. The purified protein was injected to rabbits for production of PAbs. Developed antibodies could detect the CLas infection in HLB-positive samples and did not show any cross-reactivity with the samples exhibiting nutrient deficiency symptoms (Lu et al. 2013). Sec-translocon dependent extracytoplasmic (SDEs) proteins which are specific to CLas were identified and used for development of specific PAbs (Pagliaccia et al. 2017). The specific PAbs against SDEs could effectively bind the CLas-secreted protein and detect the CLas infection in infected tissues. In previous studies, monoclonal antibodies directed against *Ca.* Liberibacter isolates from different geographical areas were shown to react with one or several isolates, but none of the antibodies reacted with all the isolates (Garnier et al. 1991; Gao et al. 1993).

The two Liberobacter species can be detected in plants and insects by DNA/DNA hybridization with probes In-2.6 for CLas and AS-1.7 for CLaf (Villechanoux et al. 1992; Planet et al. 1995). These probes contain genes of the β operon encoding, in particular, ribosomal proteins. Bove et al. (1993) and Varma et al. (1993) used In 2.6 probe for the detection of the HLB bacterium from orchard trees in India. Korsten et al. (1996) used the probe AS-1.7 to detect HLB infection in South Africa.

Recently the utilization of Fluorescence in-situ hybridization (FISH) assay with CLas-specific probe to identify the presence of CLas bacterial cells in pulverized citrus midrib extracts was reported. In this FISH assay, LSS primer (oligonucleotide) for CLas 16S rRNA gene was used as probe with fluorescein-conjugated anti-probe antibody, to confirm the rod-shaped and atypical rod-shaped spots of CLas bacterial cells in rough lemon midrib samples by fluorescent microscopy (Fujikawa et al. 2013). Furthermore, FISH utilizing the probes specific to 16S rDNA demonstrated localization of CLas bacterial cells in vascular tissues of seed coat (Hilf et al. 2013).

26.5.4 Conventional Polymerase Chain Reaction (cPCR), Amplicon Sequencing and Phylogenetic Analysis

Due to the extreme sensitivity of the test, PCR now is especially important to the citrus industry for detection of '*Candidatus* Liberibacter spp.'. Since its first discovery by Kerry Mullis in 1983 for which he was awarded the Nobel Prize in chemistry in 1993, PCR has become a powerful technique for the detection and identification of plant pathogens. The first set of 16S rDNA-based primers (OI1/O12c and OAl/O12c) was designed for conventional PCR to detect CLas and CLaf, yielding a fragment of 1160 bp in size (Jagoueix et al. 1996). A RFLP analysis of the 1160 bp PCR amplicon with restriction enzyme *Xba*I was subsequently needed to distinguish between the two Liberibacter species. CLas amplicon contains one *Xba*I restriction site, and yields two fragments (640 and 520 bp) upon restriction (Fig. 26.6), while CLaf has two such sites, and yields three fragments (520, 506 and 130 bp). Subsequently, a second set of conventional PCR primers (A2/J5) were developed based on the rplKAJL-rpoBC gene cluster (β-operon ribosomal protein gene) by Bové's group in France (Hocquellet et al. 1999; Planet et al. 1995; Villechanoux et al. 1993). This set of primers allowed for the identification of the two Liberibacter species directly by separating the different sized amplicons generated by the primers (703 bp for CLas and 669 bp for CLaf). These two primer pairs (16S rDNA- and β-operon- based) were employed in several countries for specific detection of HLB bacterium (Das 2004; Das et al. 2007; Baranwal et al. 2004, Gouda et al. 2006; Ruangwong and Akarapisan 2006; Chohan et al. 2007; Magomere et al. 2009). Since none of the primer sets above could detect the new Liberbacter species, "CLam", a new set of 16S rDNA-based primers (GB1/GB3) was developed, producing an amplicon of 1027 bp (Texeira et al. 2005a, b). Since then several pairs of conventional PCR primers (Table 26.1) have been developed mainly based on 16S rDNA and of ribosomal protein gene cluster (Jagoueix et al. 1996;

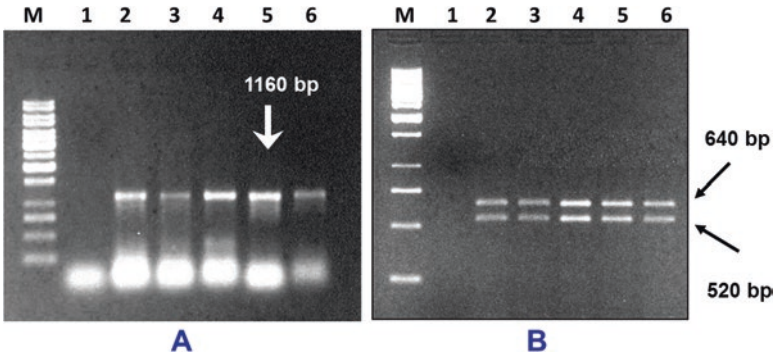

Fig. 26.6 PCR detection of HLB bacterium, CLas from citrus leaf midribs amplified with the OI1/OI2c primers (**a**), and digestion of the 16S rDNA amplified product with *Xba*I restriction enzyme (**b**). M: 1 Kb ladder lane1, extracts from healthy citrus, lanes, 2–6, extracts from CLas-infected citrus plants

Table 26.1 Primer sequences designed and developed for PCR amplification of HLB bacterium from HLB-infected plants

Primer	Sequence 5′–3′	Amplicon size (bp)	Specificity[a]	References
OI1	GCGCGTATCCAATACGAGCGGCA	1160	CLas & CLaf	Jagoueix et al. (1996)
OI2c	GCCTCGCGACTTCGCAACCCAT			
OI2c	GCCTCGCGACTTCGCAACCCAT	1500	CLaf	Jagoueix et al. (1996)
OA1	GCGCGTATTTTATACGAGCGGCA			
OL1	TCTGTTTTCTTCGAGGTTGGTGAG	563	CLas	Tian et al. (1996)
OL2	ACCGCAAGACTCCTTACCAGGAAG			
A2	TATAAAGGTTGACCTTTCGAGTTT	703	CLas	Hocquellet et al. (1999)
J5	ACAAAAGCAGAAATAGCACGAACAA	669	CLaf	
Las 266_F	CACCGAAGATATGGACAACA	226	CLas	Hung et al. (1999)
Las 266_R	GAGGTTCTTGTGGTTTTTCTG			
GB1	AAGTCGAGCGAGTACGCAAGTACT	1027	CLam	Teixeira et al. (2005a, b)
GB3	CCAACTTAATGATGGCAAATATAG			
LSg2f	TTAAGTTAGAGGTGAAATCC	545	CLam	Coletta-Filho et al. (2005)
LSg2r	CAACTTAATGATGGCAAATA			
Omp f	TAT CAT GGC CAC GGG TTA TT	809	CLas & CLaf	Bastianel et al. (2005)
Omp r	CAC GCG GAC CTA TAC CCT TA			
ITSAf	GGGGGTCGTTAATATTTGGTT	300	CLas	Deng et al. (2008)
ITSAr	GTCGCATACA ATGCCAACAT			
Las606	GGAGAGGTGAGTGGAATTCCGA	500	CLas	Fujikawa and Iwanami (2012)
LSS	ACCCAACATCTAGGTAAAAACC			
OI2	ATGGGTTGCGAAGTCGCGAGGC	800	CLas & CLaf	Jagoueix et al. (1997)
23S1	CGCCCTTCTCTCGCGCTTGA			
CN265	TGGGTGGTTTACCATTCAGT	448	CLas & CLaf	Harakava et al. (2000)
CN266	CGCGACTTC GCAACCCAT	Nested PCR with OI1/OI2c		

[a]CLas, *Ca*. L. asiaticus, CLaf, *Ca*. L. africanus, CLam, *Ca*. L. americanus

Hocquellet et al. 1999; Texeira et al. 2005a) apart from sequences from outer membrane protein (omp) gene (Bastianel et al. 2005), DNA polymerase gene (Tatineni et al. 2008; Donnua et al. 2012) and other genetic loci.

Fujikawa et al. 2013, demonstrated the successful application of direct PCR protocol for an accurate, rapid and easy to use in quarantine testing to the CLas pathogen. A combination of techniques (70% ethanol or RNAlater fixation of samples, CLas bacteria extraction with Biomasher III, and direct PCR using the Las606/LSS primer set) employed resulted in rapid detection of HLB bacterium suitable for large-scale diagnosis.

The sensitivity of detection of HLB-associated Liberibacters, has also been improved by adapting a nested PCR procedure (Deng et al. 2007; Ding et al. 2004; Li and Ke 2002). Introduction of a second pair of primers, results in amplification of the previously amplified material, leads to an increased sensitivity of detection. Nested PCR has been used to improve the sensitivity of HLB-associated Liberibacter detection in asymptomatic trees, especially in asymptomatic nursery plants, citrus relatives with low Liberibacter titer and for possible seed transmission (Benyon et al. 2008; Ding et al. 2004). The full genome sequencing of CLas (Duan et al. 2009) has facilitated researchers all over the world to evaluate other regions of the bacterium more suitable for developing a rapid and prescient PCR-based detection technologies (Morgan et al. 2012).

The identity of the Liberibacter species present in a citrus tree/ sample is generally confirmed and characterized by cloning and/or sequencing the amplicon derived from conventional PCR of the signature gene fragments (16S rDNA, ribosomal protein gene region or, any other loci). The derived sequences can further be analysed using different computer programs and online tools and then subsequently phylogenetic and evolutionary analysis could be conducted after multiple sequence alignments. Phylogenetic analysis of OI1/ OI2c DNA fragments of Katol, Kondhali and Kalmeshwar isolates collected from Nagpur mandarin growing areas of Nagpur district, Maharashtra, India revealed that the Nagpur HLB bacterium clustered together with the other CLas isolates obtained from NCBI GenBank database (Das 2009). CLaf isolates and CLam isolate formed different cluster (Fig. 26.7). The genetic distance from other members of α-proteobacteria (*Sinorhizobium* sp. *Phyllobacterium* sp. and *Rhizobium* sp.) was found even higher (Fig. 26.7).

Conventionally, CLas isolates have been characterized based on the most conserved genomic locus (16S rDNA). Due to low genetic variation in the 16S rDNA genomic region, it is not ideal target for adequate genetic resolution for categorization of strains or genotypes. Beta operon of ribosomal protein gene, 16S/23S Intergenic Spacer Region (Gupta et al. 2012) and *omp* genes (Bastianel et al. 2005; Hu et al. 2011) were alternatively used for characterizations of CLas isolates but have limited resolution potential. Single-nucleotide polymorphism (SNP) based variations in the *omp* locus suggested the grouping of CLas population from China-Guangdong with those from Thailand and Nepal, whereas the strains from Philippines and China-Behai were clustered in separate group (Deng et al. 2008). Genetic variations in the alternative loci like bacteriophage-type DNA polymerase revealed three distinct clusters for the CLas population from Southeast Asia (Tomimura et al. 2009). The clustering pattern was not in congruence with the geographical origin of isolates except for those from Indonesia. In 2009, a study was conducted to investigate the genetic diversity of CLas in Karnataka State of India in citrus trees with die-back HLB symptoms. These investigations revealed the presence of a new 'CLas' SNP genetic lineage and data was utilized to update the HLB bacterial diversity within Indian subcontinent (Adkar-Purushothama et al. 2009).

Characterization of bacterial strains or genotypes is essentially required for understanding the epidemiological patterns and for devising durable management

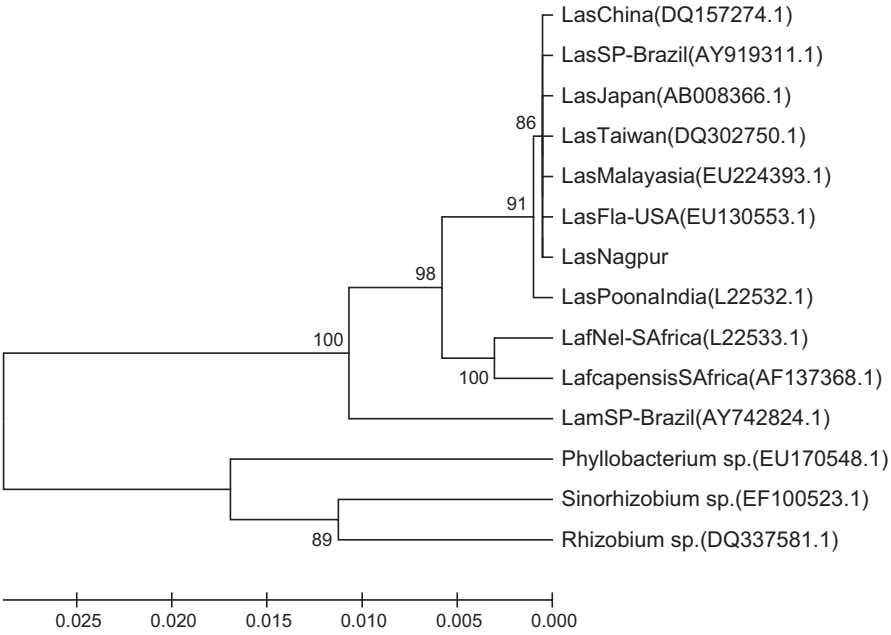

Fig. 26.7 Phylogenetic tree constructed from alignment of 16S rRNA gene sequences from *Candidatus* Liberibacter spp. and other α- proteobacteria. Genbank accession nos. are given in parentheses. Bootstrap values (based on 1000 replications) are indicated at the nodes

strategies. Limited genetic variability in the most commonly characterized genetic loci of CLas has further demanded to target the variable and hypervariable loci with adequate genetic resolution. Simple sequence repeat (SSR) or microsatellite markers with variable number of tandem repeats (VNTR) have been widely employed as robust marker in differentiating the Liberibacter species isolates at strainal level and genotypic level (Katoh et al. 2011; Ma et al. 2014;). The analysis of the tandem repeat numbers (TRNs) at the CLIBASIA_01645 locus of CLas was found to be very useful to detect and characterize the genomic variability of the HLB bacterial population (Chen et al. 2010; Ghosh et al. 2015). An analysis of a prophage terminase gene revealed genetic variations in the populations of two citrus-growing provinces in China (Liu et al. 2011). Recently, two new CLas strains were reported in California Harbor region, USA. Both strains indicated presence of different Prophages as result of different source of transmission or the occurrence of mixed CLas population (Zheng et al. 2017).

26.5.5 Real-Time PCR

A method known as "real-time PCR" (RT-PCR), also referred to as quantitative PCR (qPCR) was first reported in 1991 (Holland et al. 1991). However, when

Applied Biosystems (ABI) developed the first commercial real-time instrument in 1996 (Heid et al. 1996), real-time qPCR has become the most accurate and sensitive method for the detection and quantification of nucleic acids thus far developed. This high-throughput technique has improved the systems in use, achieving high speed, specificity, sensitivity and reliability, as well as reducing the risks of cross-contamination compared with conventional PCR method (Lopez et al. 2009). Presently in USA, official diagnostic protocol for CLas is based on RT-PCR (USDA-APHIS 2012). In 2006, species-specific TaqMan probe-primer sets, HLBaspr, HLBafpr and HLBampr were developed for detection and identification of the three known species of Liberibacter in complex PCR with the positive internal control TaqMan probe-primer set COXfpr targeting the host plant cytochrome oxidase gene (Li et al. 2006). The HLBaspr set has been successfully applied in detection, identification and quantification of "*Ca*. L. asiaticus" in host plants of citrus (Tatineni et al. 2008; Li et al. 2008) and in vector psyllids (Manjunath et al. 2007). Afterwards, based on a β-operon protein gene of a DNA isolate obtained from HLB-infected citrus in Quangxi, China, one TaqMan probe-primer set CQULA04f/r/p10 was developed specifically for detection and quantification of CLas (Wang et al. 2006). This primer pair was also utilized in the SYBR Green real-time PCR. The real-time PCR assays proved to be at least 10 to 100-fold more sensitive than the conventional PCR methods for detection of Liberibacter species associated with HLB (Li et al. 2006; Wang et al. 2006; Teixeira et al. 2008).

Studies using the primer–probe combination HLBas-HLBr-HLBp-based real time quantitative PCR (Fig. 26.8) to diagnose the presence of *Ca.* L. asiaticus in 16 citrus growing states of India have been reported (Das et al. 2014). Through RT qPCR analysis, most of the economically significant citrus varieties of mandarin, sweet orange, lime and lemon, pummelo and Satkara were found to be infected with CLas across India (Das et al. 2014). Bertolini et al. 2014 reported the development of real-time PCR methodology for direct detection of '*Candidatus* Liberibacter'

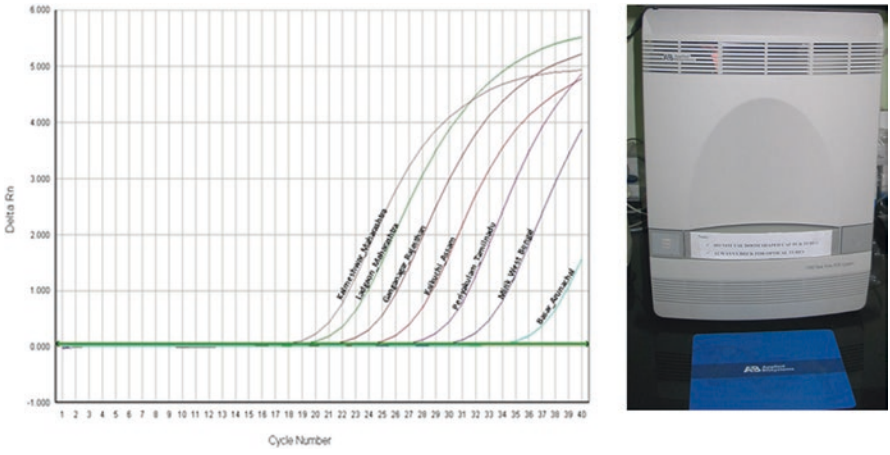

Fig. 26.8 Real time PCR detection of Huanglongbing-associated bacteria

species in tissue-printed and squashed samples of citrus plants and psyllid vectors. This methodology has been converted to a commercial kit and the intra-laboratory tests showed 96% accuracy in HLB detection (Bertolini et al. 2014). Currently, real time PCR which is precise and consistent in terms of end product is well established as a prime utility for the detection of HLB (Rigano et al. 2014).

Other real-time PCR primer-probe sets are available for CLas detection, LJ900fpr targeting hyvI/hyvII multiple tandem repeats (Morgan et al. 2012), RNR targeting nrdB gene (Zheng et al. 2016) and p3G based on 16S rDNA sequences (Orce et al. 2015). In 2014, Ángel et al. reported another efficient real-time PCR technique for the detection of 16S rDNA region of both CLas and CLam in citrus plant materials and in insect vector *D. citri*. Kogenaru et al. 2014 designed 34 qRT-PCR primers based on unique Las specific genes identified by bioinformatic analyses. Among them, 18 were found highly specific and suitable for the detection of CLas from CLas-infected plant and psyllid samples. A new diagnostic real-time PCR method for HLB detection in citrus root tissue has been described very recently (Park et al. 2018).

26.5.6 Droplet Digital PCR (ddPCR)

Droplet digital PCR (ddPCR) is a new approach that allows sensitive detection and absolute quantification of low concentration DNA without the need for an external standard curve. The PCR reaction containing DNA templates, primers and a fluorescently labeled hydrolysis probe or a nucleic acid intercalating dye is partitioned into thousands of nanoliter-sized water-in-oil droplets. A droplet with target DNA amplification shows fluorescence and is defined as positive; whereas, a non-fluorescent droplet with no target DNA is defined as negative (Pinheiro et al. 2012). The total number of target DNA molecules in a sample can be calculated from the fraction of positive droplets and Poisson statistics (Hindson et al. 2011). More recently Zhong et al. (2018) and Selvaraj et al. (2018) reported the utility of ddPCR assays to detect CLas using the 16S rRNA target in DNA extracts from citrus leaves and ACP.

26.5.7 LAMP and Lateral Flow Dipstick Method

Loop mediated isothermal amplification (LAMP) is a novel technique for the specific amplification of DNA templates at a single temperature, which does not require temperature cycling, hence requiring less specialized equipment than conventional PCR technologies. This technique has been used to detect several vertebrate and plant pathogens including bacteria and shows promise as a rapid and effective method for the detection of important pathogens (Craw and Balachandran 2012). The basic theory of LAMP is an auto cycling strand displacement DNA synthesis event which is directed in presence of specialized *Bst* DNA polymerase and a set of four to six primers. These primers recognizes six to eight regions of the target DNA to amplify the final products of stem–loop DNAs with several inverted repeats of the

target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand (Notomi et al. 2000). The end-product of LAMP (accumulated DNA product) causes either visible colour change (using visualization dye like hydroxyl naphthol blue or SYBR Green I) or also could be detected by gel electrophoresis, although gel-electrophersis makes LAMP less suitable for quarantine applications. Okuda et al. (2005) demonstrated the utilization of LAMP assay targeting a tufB-secE-nusG-rplKAJL-rpoB gene cluster for detecting HLB-associated CLas from citrus tissue. There are several reports indicating use of LAMP method to detect CLas using primers derived from the 16S rDNA (Ravindran et al. 2012; Rigano et al. 2014; Ghosh et al. 2016).

A lateral flow dipstick LAMP assay demonstrating equal efficacy with real time PCR results to detect a hypothetical protein region from CLas has been reported with several embellishments like it is target specific, no cross-reaction, no gel electrophoresis, require micro-picograms of sample with high sensitivity to detect even 10 pg of target DNA (Rigano et al. 2014). Recently an advanced loop-mediated amplification technology (LAMP) assisted with Smart-DART™ unit connected to an Android device has been developed to test psyllids for the presence of CLas (Keremane et al. 2015) and a real-time fluorescent LAMP (RealAmp) assay was also developed for the rapid and quantitative detection of CLas in China (Wu et al. 2016). Due to its simplicity in terms of instrumentation, the LAMP technology (Fig. 26.9) has several advantages such as it is economic, handy, highly responsive and speedy, and holds great promise as a field-deployable method for Liberibacter

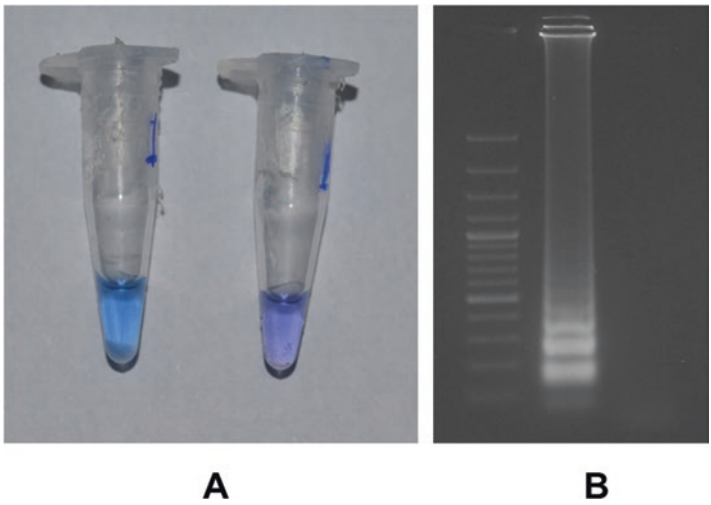

Fig. 26.9 Detection of citrus greening bacterium (*Candidatus* Liberibacter asiaticus) by LAMP. (**a**) LAMP detection using hydroxyl naphthol blue dye as a visual indicator. The reaction becomes sky blue if the target gene is present (left tube) but remains violet if the gene is absent (right tube). (**b**) Agarose gel electrophoresis of LAMP products. The positive reaction is manifested as a ladder-like pattern on the 1.5% agarose gel. M = 100 bp DNA marker. Lane1: Positive reaction (with target DNA). Lane 2: Control (Healthy citrus DNA)

detection in citrus tissues and vector psyllids (Keremane et al. 2015). However, the LAMP assay has not been validated for regulatory purposes, and there is not yet a commercially available LAMP kit for CLas detection till date (McCollum 2018).

26.5.8 Recombinase Polymerase Amplification (RPA)

RPA is another method to amplify specific DNA targets (Russell et al. 2015). It is similar to LAMP in that it is isothermal, does not require DNA purifications, expensive equipment or highly trained technicians. The entire testing process can be completed in about 30 min using a crude extract and a portable heat block. Sensitivity is reportedly comparable to that of RT-PCR (Russell et al. 2015). A commercially available RPA kit is available for Liberibacter assays (AmplifyRP® manufactured by Agdia), but is unlikely to replace qPCR for regulatory use, at least in the near future. Replacement of qPCR for confirmation of HLB associated Liberibacters would require rigorous validation for reliability, which can be a lengthy, time-consuming process. Recently Qian et al. (2018) reported an extremely rapid and simple method for field detection of CLas from leaf samples, based on recombinase polymerase amplification (RPA) that can be accomplished within 15 min.

A comparative account of different PCR-based assays currently used to detect '*Candidatus* Liberibacter spp.' has been described in Table 26.2.

26.5.9 Use of Spectroscopic and Imaging Techniques

Specific regions in the electromagnetic spectra have been realized to provide information about the physiological stress in plants, and accordingly, diseased plants usually display diverse spectral signature than non-stressed healthy plants in those specific ranges. The spectral reflectance from the tree canopy in the visible and infrared regions of the electromagnetic spectra can be used as a sign of plant stress (Sankaran et al. 2010). Differences in the spectral reflectance of healthy and diseased plants can be seen in the visible–infrared region (Purcell et al. 2009). Spectroscopy in the range of visible and near infrared has been examined for disease detection in a great variety of crops, since it is a rapid and non-destructive tool that can be used in real-time crop assessment under field conditions (Sankaran et al. 2010). Assessment of spectral features in visible-near infrared spectroradiometer spectra for the specific detection of HLB disease was reported by Sankaran et al. (2011). This study utilized the two spectral features (i) spectral reflectance bands and (ii) vegetation indices (VIs) along with two classifier models which in totality indicated HLB detection accuracy more than 80%.

A study was conducted in Florida in 2007 and 2009 to detect HLB infected trees based on multispectral (MS) and hyperspectral (HS) airborne images of citrus groves (Kumar et al. 2012). These studies clearly depicted that the healthy canopy had higher reflectance in the visible range, and lower reflectance in the near-infrared (NIR) range than HLB infected canopy. In this study disease density maps were also

Table 26.2 Comparison of various PCR-based assays used to detect '*Candidatus* Liberibacter spp.' in citrus trees and psyllid vectors

Technique	Conventional PCR (cPCR)	Real-time PCR (rtPCR)	Droplet digital PCR (ddPCR)	Loop mediated isothermal amplification (LAMP)	Recombinase polymerase amplification (RPA)
DNA extraction	Similar for citrus and psyllid			Crude extract	Proprietary
	Various methods based on three steps:			Pulverise sample	
	1. Pulverize sample			Incubate 10 min also amenable to more sophisticated amplification protocols	
	2. Extract soluble material				
	3. Fractionate soluble material into DNA and non-DNA				
	DNA extraction kits are typically used due to consistency and capacity for high throughput PCR can be affected by inhibitors present in the DNA preparation.				
Necessary pre-amplification steps	None	None	Droplet generation	None	None
Amplification requirements	Thermocycler	Real-time thermocycler	Thermocycler	Constant temperature (heat block or water bath)	Constant temperature (heat block)
Detection methods	Post amplification	During amplification	Post amplification	Either during amplification or post amplification	Visual
	Visual	Probe based	Fluorescence	Fluorescence	Dip stick (+/− test result)
	Fluorescence/ uv light	Fluorescence/ laser	Droplets classified as positive or negative	+/− test result	
	Manual analysis	Automated analysis	Quantification based on statistical computation		
		Ct value is calculated by software, proprietary algorithms			
Sensitivity (number of bacterial cells per reaction)	100	1	1	100	10
Field use	No	No	No	Potentially	Yes

Modified after McCollum (2018)

generated from the classification results and were observed to be helpful to identify the severely infected areas. Sankaran and Ehsani (2012) demonstrated the potential of using handheld fluorescence sensing for stress detection in citrus leaves, especially HLB detection in citrus. Results of another study revealed the efficient application of visible-near infrared and thermal imaging for detection of HLB disease in citrus trees (Sankaran et al. 2013). Therefore, in future ground scouting aided with spectroscopic and airborne spectral imaging techniques would certainly prove to be an asset for managing HLB disease with imaging. By evaluating the ability of narrow-band imaging and polarizing filters in detecting starch accumulation in symptomatic citrus leaf. Pourreza et al. (2014) showed that the starch accumulation in HLB-symptomatic leaves rotated the polarization planar of light at 591 nm, and this property can be effectively used in a fast and low-cost HLB detection system in field conditions.

Detection of HLB through optical sensing has also been explored but this technology is still being optimized and tested. Field experiments have demonstrated that optical sensors, such as a rugged, inexpensive multi-band active sensor that measures reflectance of tree canopy in two visible bands at 570 nm and 670 nm, and two near infrared bands at 870 nm and 970 nm (Mishra et al. 2011); and a field portable spectroradiometer that collects the spectral reflectance data in the range of 350–2500 nm (Sankaran and Ehsani 2011) can be used to identify HLB-infected trees from healthy trees. Differentiation of infected and healthy trees based on tree canopy reflectance was also achieved using airborne multispectral and hyperspectral images (Kumar et al. 2012). Unmanned aerial vehicle (UAV) imaging in combination with remote sensing and support vector machine produced higher classification accuracies than images obtained from aircraft (Garcia-Ruiz et al. 2013).

26.5.10 Biomarkers and Profiling of Plant Volatiles

Substantial progresses have been made in HLB detection technologies, particularly those that detect biomarkers produced by the infected host: a biomarker secreted by the pathogen in the host, or volatile organic compounds (VOCs) released by an infected host (Cevallos-Cevallos et al. 2011, 2012; Aksenov et al. 2014; Ding et al. 2017; Pagliaccia et al. 2017; Slupsky 2017). Detection of HLB infected plants based on plant volatiles is a upcoming and promising strategy which is based on the chemical analysis of VOCs that are released by HLB-infected trees. Biomarkers specific to CLas have been found and could be analysed using gas chromatography-mass spectrometry (GC-MS) and gas chromatography/differential mobility spectrometry (Aksenov et al. 2014). Greenhouse tests revealed that a mobile differential mobility spectrometry system was able to distinguish VOC differences between closely-related citrus cultivars and show volatile-profile differences between healthy and infected citrus (McCartney et al. 2016). Recently in a study conducted in Mexico, the compounds: D-limonene, β-ocimene, and caryophyllene were found at higher concentrations in the HLB infected young shoots of Persian and Mexican lime (Mendoza-Peña et al. 2018). The use of canines/dogs to sniff out these volatiles

specific to infected trees has been an effective strategy for citrus canker detection and preliminary observations show promise for dogs to be able to detect HLB infections before symptom appearance (Berger 2014).

26.6 Concluding Remarks

Symptoms associated with HLB in citrus plants, although well-known and distinguishable in many cases, can under certain circumstances induce doubts on recognition of diseased trees. For this reason, diagnostic methods based either on the reaction of the plant or on the identification of the associated pathogen are necessary. The integrated diagnostic and management system is the utmost need of an hour to eradicate the threat of HLB to the worldwide citrus industry. In this paper we have summarized the information regarding tools and techniques available for diagnosis and detection of HLB and the associated bacterium '*Ca.* Liberibacter' in citrus trees or in vector insects. Tools and techniques such as microscopic analysis and identification of causal agent, molecular markers and PCR-based diagnostics, spectroscopic screening, isothermal amplification aided with advanced technology of lateral flow dipstick or Smart-DART™, analytical techniques for profiling plant volatile organic compounds and visual sensors and imaging systems were discussed. All these techniques when used in combination could have a promising edge over the singular application towards efficient detection and management of HLB.

The most critical and time-sensitive diagnostic need is to find and validate methods of detecting infection during the latency period when CLas titers are, for the most part, undetectable. These will be methods that indirectly detect the disease (i.e., HLB) rather than the CLas pathogen. Exploration of the use of biomarkers should continue, with comparisons of omics biomarkers and VOCs. Presently, real time qPCR followed by conventional PCR and DNA sequencing are the approved methods that must be used by laboratories accredited under the respective National Plant Protection Programmes of the citrus growing countries around the globe. Nonetheless, it is also essential for the researcher to carry on more in-depth studies regarding optimizing and refining the existing HLB diagnostic tools and detection techniques to create a novel, economic and accurate management policy directed towards minimising HLB-induced economic losses.

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Chloroplast Proteins and Virus Interplay: A Pathfinder to Crop Improvement

27

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Abstract

Plant viruses always posed extensive losses to crop production. Thus, it is of utmost importance to plant virologists and biologists to accurately identify culprit host plant proteins which participate in plant-virus interactions. Advancements in molecular virology and plant biotechnology have led to many major breakthroughs in past years enabling recognition of innumerable host factors of virus-plant interactions. Interestingly majority of these host factors are chloroplast and photosynthesis related proteins. Hence chloroplast-virus interaction is an epicentre of plant-virus interplays, and its study could help to understand mechanisms of virus infection, spread, symptom development and host resistance. Advanced proteomic tools have empowered the development of sensitive and effective methods to detect host and viral proteins of interplays. Thus, precise information on chloroplast-virus interaction could be used to develop finer disease control strategies and genetically engineered plants with better photosynthetic efficiency and yields.

Keywords

Chloroplast-virus interaction · Plant defence · Plant-virus interaction · Viral movement · Viral replication · Virus symptoms

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27.1 Introduction

Plant viruses are simple obligate intracellular biotrophic or necrotrophic pathogen. Simplest viruses are composed of a small genome (RNA or DNA) surrounded by a coat protein (CP) (Yadav and Khurana 2016). Viruses are entirely dependent on host cellular machinery for infection cycles *viz.* Viral replication, intracellular and cell-to-cell movement (Nelson and Citovsky 2005). Viruses are catastrophic and virus infections in plants result in reduced plant growth, lower yield and inferior quality of produce, leading significant loss to crop production (Kumar et al. 2010; Yadav and Khan 2008; Yadav and Khurana 2016).

As a consequence of virus diseases plants exhibit altered morphology and physiology as symptoms. Host and virus genotypes as well as environmental conditions collectively impact host physiology thereby influence nature and degree of symptom incidence in host. Symptoms production and disease development are outcome of complex cellular, sub-cellular and supra-cellular virus-plant interactions (Revers et al. 1999). Most notable symptoms induced by plant viruses include stunting, yield losses and reduction in life span of diseased host. Symptoms of virus infection usually appear on leaves e.g. potyviruses like *Narcissus yellow stripe virus* (Yadav and Khan 2015); additionally prominent symptoms may appear on stem, fruits and roots (Shukla et al. 1994).

Leaf chlorosis is one of the most prevalent symptoms by plant virus due to alteration of host's structure and function. Plants with virus induced loss of chlorophylls and other pigments exhibit mosaic, chlorotic or necrotic streaks, chlorotic vein banding, mosaic, distortion of leaves, etc.

Chloroplast is most common target of virus infection as it harbours proteins and enzymes for anabolic processes *viz*. photosynthesis and biosynthesis of majority of plant hormones. Chloroplast factors are crucial in plant defence response and cross-talk among organelles. Carotenoids found in chloroplast membranes protect photosynthesis against photo-damage as well as potentially enhances nutritional quality and yield. Also, chloroplast affects plant development via its roles in abiotic and biotic stress signalling (Ashraf and Harris 2013).

27.2 Viral Disease Symptoms & Intervention of Chloroplast

Studies on different plant viral diseases and causal agents revealed that secretes to majority of devastating diseases are hidden in ssRNA or ssDNA viral-genomes (Yadav and Chhibbar 2018) that bring structural and functional anomalies in chloroplast on infection.

Roberts and Woods (1982), using electron microscopy reported that *Cucumber mosaic virus* (CMV) strain-P6 induces severe chlorosis in *Nicotiana tabacum* on systemic infection and alters chloroplast structure instead of chloroplast disruption and excess starch accumulation. Photosynthesis associated symptoms like chlorosis and mosaic occur due to abnormal chloroplast function such as altered polypeptide

Table 27.1 Virus and chloroplast interplay in symptom production

Virus	Virus components	Chloroplast factors	References
Alternanthera mosaic virus (AltMV)	TGB3	PsbO	Jang et al. (2013)
Potato virus X (PVX)	CP	Plastocyanin	Qiao et al. (2009)
Cucumber mosaic virus strain M (CMV-M)	CP	Fd I	Qiu et al. (2018)
Cucumber mosaic virus strain Y satellite RNA (CMV-Y-sat)	22-nt vsiRNA (virus derived interfering RNA)	ChlI mRNA	Shimura et al. (2011) and Smith et al. (2011)
Potato virus Y (PVY)	CP	RbCL	Feki et al. (2005)
	HC-Pro	MinD	Jin et al. (2007)
	HC-Pro	CF1β	Jin et al. (2007)
Sugarcane mosaic virus (SCMV)	HC-Pro	Fd V	Cheng et al. (2008)
Soybean mosaic virus (SMV)	P1	Rieske Fe/S	Shi et al. (2007)
Tomato mosaic virus (ToMV)	CP	Ferredoxin	Sun et al. (2013) and Ma et al. (2008)
Rice stripe virus (RSV)	SP	PsbP	Kong et al. (2014)

expression of oxygen-evolving complex in thylakoid membranes (Rahoutei et al. 2000). Leaf mosaic symptom on virus infection occurs by clustered arrangement of mesophyll cells containing damaged chloroplasts (Almási et al. 2001). *Potato virus* Y (PVY) symptoms are produced due to reduced number and size of chloroplasts and decreased photosynthesis in host tissues (Pompe-Novak et al. 2001).

Various plant viral proteins involved in virus symptom production in host plants and their interacting counterparts in chloroplast are summarized in Table 27.1.

27.2.1 Virus Generated Anomalous Structures in Chloroplast

Virus infection induces structural changes in chloroplast and reduces abundance of photosynthetic proteins. Chloroplast has multifaceted biological functions and is prone to structural changes by biotic and abiotic stresses (Jin et al. 2018). Broad range of chloroplast malformations are induced by viral attack that involves variety of viral factors as summarized in Table 27.2 and some frequently observed chloroplast structural changes are as follows (Zhao et al. 2016a):

(i) Clustering and reduced number of chloroplast.
(ii) Globular or amoeboidal shaped chloroplast.
(iii) Stromule formation i.e., dynamic thin protrusions of envelope membrane.
(iv) Abnormal vesicle or cytoplasmic invagination to exterior of outer chloroplast envelope.

Table 27.2 Virus induced anomalous structures in chloroplast

Virus	Host	Structural malformation	References
Abutilon mosaic virus (AbMV)	*Abutilon sp.*	Disorganization of thylakoid system; complete elimination of grana-stroma	Schuchalter-Eicke and Jeske (1983)
		Degeneration of lamellar system; its substitution by amorphous electron-dense material	Gröning et al. (1987)
	Nicotiana benthamiana	Induction of stromules	Krenz et al. (2012)
Alternanthera mosaic virus (AltMV)		Vesiculation at chloroplast membrane	Lim et al. (2010)
Artichoke mottled crinkle virus (AMCV)	*Chenopodium quinoa*	Distorted grana and paired membranes	Martelli and Russo (1973)
Barley stripe mosaic virus (BSMV)	*Hordeum vulgare*	Vesiculated chloroplast with vesicle located between inner and outer membrane, virons surrounded chloroplast, cytoplasmic invaginations into chloroplasts with virons; rearrangement of thylakoids, electron transparent vacuoles in stroma	Carroll (1970); Zarzyńska-Nowak et al. (2015)
	Datura stramonium	Swelling, deformation of membranes, cytoplasmic invaginations, peripheral vesicles, virus particles attached to limiting membrane, pellucid appearance of stroma	McMullen et al. (1978)
	Nicotiana benthamiana	Virus associated with vesicles and cytoplasmic inclusions of chloroplast	Torrance et al. (2006)
		Envelope membranes forming peripheral invaginations and spherules; presence of large cytoplasmic invaginations	Jin et al. (2018)
Bean yellow mosaic virus (BYMV)	*Vicia faba*	Increased stromal area, total or partial loss of envelopes, dilated grana and thylakoids, reduced chloroplast number	Radwan et al. (2008)
Beet western yellows virus (BWYV)	*Lactuca sativa, Claytonia perfoliata*	Disappearance of grana stacks, stroma lamellae, increased starch, increase in number & size of plastoglobuli	Tomlinson and Webb (1978)
Belladonna mottle virus physalis mottle strain (BeMV-PMV)	*Datura stramonium*	Development of vesicles in chloroplasts, vesiculations of the outer membranes of chloroplast	Moline 1973

(continued)

Table 27.2 (continued)

Virus	Host	Structural malformation	References
Broad bean wilt virus 2 isolate B935	*Vicia faba*	Inhibited lamellar development, membrane vesiculation	Li et al. (2006)
Broad bean wilt virus 2 isolate PV131		Chloroplast with swollen or disintegrated membrane	
CMV isolate 16	*Lycopersicon esculentum*	Chloroplast damage and malformation including: Reduction in chloroplast number and size, chloroplasts later completely destroyed and disorganized grana scattered into the cytoplasm, reduction in chlorophyll contents	Montasser and Al-Ajmy (2015)
CMV Malaysian isolate	*Catharanthus roseus*	Large starch grains, disorganized thylakoid system, crystalline array of phytoferritin macromolecules	Mazidah et al. (2012)
CMV P6 strain	*Nicotiana tabacum*	Reduced chloroplast size, fewer grana	Roberts and Wood (1982)
CMV pepo strain with CP_{129} substitutions		Few thylakoid membranes and granal stacks	Mochizuki and Ohki (2011)
CMV pepo strain VSR deficient mutant with CP_{129} substitutions		Fewer thylakoid membranes	Mochizuki et al. (2014a)
Maize dwarf mosaic virus strain A (MDMV-A)	*Sorghum bicolor*	Presence of small vesicles, deformation of membranes, reduction in granal stack height, disappearance of osmiophilic globules and degeneration of structures	Choi (1996)
MDMV Shandong isolate (MDMV-SD)	*Zea mays*	Chloroplasts were heavily disrupted, including thylakoid swelling and envelope broking	Guo et al. (2004)
Maize necrotic streak virus (MNeSV)		Deformed chloroplast	De Stradis et al. (2005)
Maize rough dwarf virus (MRDV)		Swelling of grana discs, rupture of the chloroplast membrane, rupture of plastidial membrane and plastidial degeneration on.	Gerola and Bassi (1966)
	Chenopodium quinoa	Distorted grana and paired membranes	Martelli and Russo (1973)
Melon rugose mosaic virus (MRMV)	*Cucumis melo*	Chloroplast alterations with peripheral vesicles and tendency to aggregate	Mahgoub et al. (1997)

(continued)

Table 27.2 (continued)

Virus	Host	Structural malformation	References
Plum pox virus (PPV)	*Prunus persica*	Dilated thylakoid membranes, increased number and size of plastoglobuli, decreased amount of starch	Hernández et al. (2006)
		Decreased starch content, disorganized granal structure	Clemente-Moreno et al. (2013)
	Pisum sativum	Dilated thylakoids, increase in number of plastoglobuli, decreased starch content	Díaz-Vivancos et al. (2008)
Potato mop-top virus (PMTV)	*Nicotiana benthamiana*	Abnormal chloroplasts with cytoplasmic inclusions and terminal projections, large starch grains in infected plastids	Cowan et al. (2012)
Potato virus S (PVS)	*Chenopodium quinoa*	Presence of osmiophilic globules, cytoplasmic invagination of chloroplasts	Garg and Hegde (2000)
Potato virus X (PVX)	*Datura stramonium, Solanum tuberosum*	Cytoplasm invaginations into chloroplast	Kozar and Sheludko (1969)
	Nicotiana benthamiana	Dilated grana	Qiao et al. (2009)
Potato virus Y (PVY)	*Solanum tuberosum*	Chloroplasts swelling, loosening of thylakoid structure, changed optical density of chloroplasts, increased number of chloroplasts, smaller chloroplasts with exvaginations	Pompe-Novak et al. (2001)
	Nicotiana sp.	Disrupted chloroplast development	Naderi and Beger (1997)
		Decreased starch, increased amount of plastoglobuli	Schnablová et al. (2005)
Ribgrass mosaic virus (RMV)		Anomaly in number, size, shape and clumping pattern of chloroplasts, presence of large starch grains, osmophilic globules	Xu and Feng (1998)
Rice stripe virus (RSV)	*Oryza Sativa*	Reduced sheets of grana stacks, increased amount and size of starch granules	Zhao et al. (2016b)
	Nicotiana benthamiana	Membrane proliferations	
Sugarcane mosaic virus (SCMV)	*Sorghum bicolor*	Swollen chloroplast, increased number of plastoglobuli	El Fattah et al. (2005)
Sugarcane Yellow Leaf Virus (ScYLV)	*Saccharum* sp.	Swollen chloroplast, rectangular grana stacks, more plastoglobules	Yan et al. (2008)

(continued)

Table 27.2 (continued)

Virus	Host	Structural malformation	References
Tobacco mosaic virus (TMV)	*Lycopersicon esculentum*	Aggregates and vacuoles in chloroplast	Shalla (1964)
		Enlarged plastids, supergranal thylakoids, large accumulations of osmiophilic bodies	Arnott et al. (1969)
	Capsicuum anuum	Swelling, more osmophilic plastoglobuli, loosened thylakoid structure	Mel'nichuk et al. (2002)
	Nicotiana tabacum	Anomaly in number, size, shape and clumping pattern of chloroplasts, presence of large starch grains, osmophilic globules	Xu and Feng (1998)
TMV flavum strain		Swollen to nearly or fully globular chloroplasts; distorted thylakoid membranes; depletion of grana; large starch grains	Lehto et al. (2003)
TMV U5 strain		Peripheral vesiculation	Betto et al. (1972)
TMV yellow strain	*Solanum tuberosum*	Disintegration and degradation of chloroplast, osmiophilic globules	Liu and Boyle (1972)
Tomato bushy stunt virus (TBSV)	*Gomphrena globosa*	Chloroplasts severely altered & often connected to multivesicular bodies, plastidal vesicles & vacuoles containing fibrillar material	Appiano et al. (1978)
	Datura stramonium	Large inter-membranous sac, complete rearrangement of thylakoids	Bassi et al. (1985)
Tomato mosaic virus (ToMV)	*Nicotiana tabacum*	Severe destruction & deformation of chloroplasts; formation of discrete dark-staining materials adjacent to plastids, large starch grains	Ohnishi et al. (2009)
ToMV L11Y strain		Flaccid chloroplast, reduced thylakoid stacks and enlarged spaces between stacks, penetration of cytoplasm into chloroplast, tubular complexes	
Tomato spotted wilt virus (TSWV)		Peripheral vesicles	Mohamed (1973)
Turnip mosaic virus (TuMV)	*Chenopodium quinoa*	Chloroplast aggregation, of irregular shaped chloroplast, large osmiophilic granules, poorly developed lamellar system, few or no starch grains	Kitajima and Costa (1973)

(continued)

Table 27.2 (continued)

Virus	Host	Structural malformation	References
Turnip yellow mosaic virus (TYMV)	*Brassica rapa*	Small peripheral vesicles; reduction in number & size of grana, chlorophyll content, starch content; increased amounts of phytoferritin & number of osmiophilic globules	Ushiyama and Matthews (1970) and Hatta and Matthews (1974)
Wild cucumber mosaic virus (WCMV)	*Marah oreganus*	Chloroplasts surrounded by single membrane vesicles; numerous double membrane-bound vesicles in chloroplast	Allen (1972)
Zucchini yellow mosaic virus (ZYMV)	*Cucurbita pepo*	Chloroplast number decreased; amount of plastoglobuli & starch increased; amount of thylakoids decreased	Zechmann et al. (2003)

(v) Inter-membrane sac formation alters plastid content *viz*. increased number and size of electron-dense granules or plasto-globules, starch grains and stromal vesicles.
(vi) Lack of stroma or grana stacks and distorted or dilated thylakoid.
(vii) Completely destroyed chloroplasts and disorganized grana scattered in cytoplasm.

27.2.2 Viral Components Evoke Anomalous Structures in Chloroplast

Viral attack affects chloroplast ultrastructure and symptom development via interaction of viral structural (CP) and/or functional factors with host proteins. Role of CP in chlorotic or white mosaic symptom development and PVY infection was established by transformation of tobacco plants separately with PVY-CP(control) and chimeric construct PVY-CP-TP (PVY-CP fused to transit peptide or TP). Pea ribulose bisphosphate carboxylase small subunit used as TP targeted CP to stroma of chloroplast. Chlorotic symptoms appeared only in PVY-CP-TP and not in PVY-CP transformed plants. In chimeric transformants CP was imported to chloroplast and it associated with thylakoid membranes; and disrupted chloroplast development (Naderi and Beger 1997).

Tobacco rattle virus localizes inside strongly disorganized chloroplasts and mitochondria of infected potato and tobacco leaf tissues (Otulak et al. 2015). BSMV induces abnormal peripheral invaginations and spherules formation from envelope membranes and large cytoplasmic invagination in reconstructed chloroplasts while healthy tobacco chloroplast lacked such structures (Jin et al. 2018). These peripheral invaginations remain in continuation with inner but not the outer chloroplast membrane. Spherules formed in inner membrane invaginated packets serve as site for BSMV replication and viral αa replication protein localizes at chloroplast surface which elicits rearrangement of chloroplast membrane.

27.2.3 Regulated Expression of Chloroplast Genes on Virus Invasion

In addition to constitutively expressed housekeeping genes, plants also have regulated genes exhibiting altered expression under biotic and abiotic stresses. Such differential expression of genes in host plants may also be regulated by generic and specific virus-plant interplay. Chloroplast is main target for virus invasion hence, suffers altered expression of genes encoding chloroplast and photosynthesis related proteins. Fine-tuning of gene regulation occurs at transcription or translation levels or half-life of transcripts or proteins thus, affecting availability of biologically active proteins and resulting in symptom development (Zhao et al. 2016a). So, study of altered expression of chloroplast proteins at transcription and translation, provides comprehensive understanding of molecular phenomenon of virus infection and symptom development. Key metabolic processes that occur in plastids are amino acid biosynthesis, Calvin cycle, carbohydrate metabolism and photosynthesis (Kleffmann et al. 2004). Establishment of virus infection involves altered expression of chloroplast resident or chloroplast membranes associated proteins. RSV infection in rice down-regulates expression of photosynthesis associated chloroplast genes which strongly control symptom development (Cho et al. 2015). Such down-regulation of host genes can significantly be correlated to degree of chlorosis (Dardick 2007; Mochizuki et al. 2014b).

Dardick (2007), identified 198 plastid related genes exhibiting altered expression on ToRSV infection out which 181 were down-regulated which included genes with functions in chloroplast (STMIN38), chlorophyll (STMEJ57), starch degradation (STMGJ02) and inhibition of chlorophyll biosynthesis (STMIL23). While, on PPV infection 137 plastid genes exhibited altered expression among which 113 were down-regulated. Moreover, both ToRSV and PPV infections down-regulated 83 nuclear-encoded plastid related genes with functions in Calvin cycle, light harvesting, and photosynthesis.

CP is one of major virulence determinant of plant viruses and amino acid substitutions in CP may alter degree of symptom production. Comparative transcriptome analyses of healthy and diseased tissues infected with pepo strain and two CP mutant strains of CMV showed varied degree of chlorosis. Functional genomic analyses of CMV infected host exhibited down-regulation of chloroplast- and photosynthesis-related genes while up-regulation of many nuclear encoded abiotic stress responsive genes (Mochizuki et al. 2014b).

Babu et al. (2008) using microarray analysis identified 2013 and 1457 fairly up- and down-regulated *Arabidopsis* genes in PPV-infected leaves, respectively, with gene functions in virus infection and symptom development. The up-regulated genes were associated with soluble sugar, starch, amino acid metabolism, intracellular membrane, chloroplast and protein fate, while down-regulated genes were associated with translation, development or storage proteins and cell wall components. PPV induced up-regulation of 52 *Arabidopsis* chloroplast genes i.e. 60% of total chloroplast genes, associated with mosaic and chlorosis symptoms.

TuMV infection in *Arabidopsis* down-regulates 30 chloroplast genes with functions in light harvesting, photosynthesis, sulphur assimilation and starch metabolism. Among which proteins encoded by 11 genes, localized in chloroplast with functions *viz*., sulphate utilization or cell wall expansion. Such differential expression of genes brings virus accumulation and symptoms development (Yang et al. 2007).

Beta-satellite associated with radish leaf curl disease in tobacco down-regulated host genes associated with chlorophyll biosynthesis, chloroplast development and plastid translocation while genes related to chlorophyll degradation were unaffected (Bhattacharyya et al. 2015).

Geminiviral protein, AC2both full-length and truncated alters expression of 834 *Arabidopsis* genes in early infection. AC2 mediated inactivation of SnRK1.2 protein, alters expression of 499 host genes. Geminivirus induced differentially expressed chloroplast transcriptome elicits defence responses by SA and JA biosynthesis in chloroplast (Liu et al. 2014).

RSV infection selectively alters the expression of rice miRNA associated with a wide range of cellular functions such as integral components of membrane, chloroplasts, nucleus, ATP binding, ubiquitin protein ligase binding, zinc ion binding, transcription, hormone signalling and defence responses (Yang et al. 2016).

The sRNAome and transcriptome analyses of grapefruit co-infected with *Citrus tristeza virus* (CTV) and *Citrus dwarfing viroid* (CDVd), spotted many differentially regulated chloroplast associated genes which influence photosynthesis. Expression of chloroplastic Magnesium-chelatase subunit ChlH (CHLH) is inversely regulated compared to its regulating sRNA. *Arabidopsis* CHLH helps in chlorophyll biosynthesis, expression of photosynthesis-related proteins and ABA signalling. Thus, disease and symptom development on CTV and CDVd co-infection is intricately associated with chloroplast (Visser et al. 2017).

Viruses either activate or suppress host gene expression for successful infection. Thus, profiling of altered plant gene expression may provide better insights into the molecular mechanisms governing host physiology and phenotype in response to virus infection.

27.3 Role of Chloroplast in Viral Infection Cycle

Plants transport endogenous macromolecules like proteins, nucleic acids across cellular boundaries in controlled manner. Viruses utilize host's intra- and intercellular pathways for their systemic spread within plant. Virus and chloroplast interplays significantly govern virus multiplication and movement.

Gemini-viruses on systemic infection induce various ultrastructural changes in chloroplast *viz*. vesiculation, reduced starch and chlorophyll content, accumulation of fibrillar inclusions, virus-like particles and viral DNA within plastids (Krenz et al. 2012). Plant viral components target organelles like chloroplasts for escalated utilization of host cellular machineries for viral-replication and spread in

susceptible host. Viruses alter shape, composition, function, and dynamics of organelles during viral-replication cycle. Membrane bound organelles have unique organic and inorganic makeup hence perform specialized functions. Structural and functional regulation of organelles is a key to maintain cellular homeostasis. Viruses utilize specialized functions of organelles and modify organelle composition for activation of viral components to target sub-cellular locations during virus cycle (Jean Beltran et al. 2017).

27.3.1 Intervention of Chloroplast Proteins in Virus-Replication

Plant viruses infect host, re-programme host metabolic activities and suppress host defence responses. Viral-replication involves interactions between various biomolecules from viruses and plants *viz*. protein-protein, RNA-protein and protein-lipid. VRC formation at replication site takes place by assembly of viral replicase, virus-encoded accessory proteins and host factors to recruit components of VRC (Panavas et al. 2005).

Replication of positive-strand RNA viruses is viral replicase dependent process which occurs on surfaces of cytoplasmic membranes (Ahlquist 2002). Genomic RNA of TYMV encodes two replication proteins *viz.* 140K and 66K. The 140K protein possess methyltransferase, proteinase and NTPase/helicase activities, whereas, 66K protein is a RNA-dependent RNA polymerase (RdRp). Confocal microscopy using antibodies against these proteins showed 140K dependent co-localization of 66K in VCR (Jakubiec et al. 2004).

Positive stranded RNA viruses on infection form cytoplasmic membrane-bound factories for viral-genome multiplication. Potyviral 6K2 is integral membrane protein that induces formation of ER-derived replication vesicles which in-turn targets chloroplasts for viral-replication. In TuMV infection 6K2 vesicles are transported and accumulated at chloroplast where they induce formation of chloroplast-bound elongated tubular structures and ultimately form chloroplast aggregation (Wei et al. 2010). Plant viruses co-opt diverse host proteins for viral-replication which may perform similar tasks in viral-replication. Positive-sense RNA viruses exploit host factors *viz*. RNA-binding proteins for viral RNA replication; proteins associated with membrane bending to form membrane-bound VRCs; lipid biosynthesis enzymes to alter lipid composition creating virus-replication supportive cellular environment; and chaperones for proper folding of viral-replication proteins to form VRCs (Nagy and Pogany 2012). The P6 protein of *Cauliflower mosaic virus* (CaMV) has a range of functions and it interacts with chloroplast proteins and most abundant in VRCs (Hohn et al. 1997). During viral infection, RNA helicase protein, ISE2 (increased size exclusion limit 2), is highly expressed and accumulated in chloroplast and is putatively involved in viral replication and spread (Ganusova et al. 2017).

Various chloroplast factors interact with viral components during viral-replication (Table 27.3).

Table 27.3 Chloroplast factors and their viral counterparts in virus replication

Plant virus	Virus components	Chloroplast factors	Subcellular localization	References
Bamboo mosaic virus (BaMV)	RNA 3′ UTR	cPGK	Chloroplast cytoplasm,	Cheng et al. (2013)
Alfalfa mosaic virus (AMV)	CP	PsbP	Cytoplasm	Balasubramaniam et al. (2014)
Cucumber mosaic virus (CMV)	1a, 2a	Tsi1- interacting protein 1	Cytoplasm	Huh et al. (2011)
Potato mop-top virus (PMTV)	TGB2	Chloroplast lipid	Chloroplast	Cowan et al. (2012)

27.3.2 Intervention of Chloroplast Proteins in Virus Trafficking Within Host

Viral factors hijack and interact with chloroplast factors for viral movement and to alter chloroplast function. Specifically regulated transport of endogenous macromolecules is utilised by plant viruses for inter/intracellular trafficking and spread of viral proteins, DNA or RNA across cellular boundaries within host. Thus, successful trafficking of virus across plant cells is essential for systemic spread and infection. Targeting of viral nucleoprotein complexes and MPs to plasmodesmata involves interaction cytoskeleton/endomembrane system of plant cell with viruses (Krenz et al. 2012). Virus Systemic infection is resulted due to intercellular movement of virus across plant cells via symplastic routes which require viral-genome encoded MPs (Zhao et al. 2016a). MPs share some characteristics to support systemic spread *viz*. nucleic acid binding activity (Citovsky et al. 1990), specific localization to plasmodesmata (Ding et al. 1992; Fujiwara et al. 1993) and ability to increase size exclusion limit of plasmodesmata (Wolf et al. 1989).

In planta vesicle trafficking and fusion is facilitated by SNAREs, a protein superfamily. Wei et al. (2013) screened ER-localized SNAREs or SNARE-like proteins for their role in TuMV infection. During TuMV infection 6K2 viral protein vesicle are trafficked to chloroplast, followed by its accumulation and formation of chloroplast-bound elongated tubular structures after chloroplast aggregation via functional actomyosin motility system. A knockdown study on Syp71, a plant protein that co-localizes with chloroplast-bound 6K2 complex, is essential for TuMV infection by virus-induced fusion of vesicles with chloroplasts.

Viral CP and TGB proteins accompany intercellular trafficking of potexviruses. TGB3 protein of AltMV accumulates in host mesophyll cells while that of PVX in ER. TGB3 has internal signal essential for its chloroplast-localization. Site-specific mutation of chloroplast-localization signal in AltMV-TGB3 disabled, virus intercellular movement from epidermis to mesophyll layer. Thus, show that suggesting mesophyll targeting by chloroplast-localization signal is essential for systemic viral-movement within host (Lim et al. 2010).

Cell-to-cell movement of AbMV, a geminivirus occurs by "couple-skating" model. AbMV hijacks various cellular pathways for intracellular transport and to target plasmodesmata by alternate routes through chloroplasts and stromules using plastidal chaperone. AbMV-MP interacts with chloroplast targeted 70-kD heat shock protein (Hsp-70) and co-localized to chloroplasts. Silencing of Hsp-70 gene affects chloroplast stability and reduced AbMV movement while viral-DNA accumulation remained unaffected. Thus, plastidal Hsp-70 is crucial for AbMV trafficking to adjoining cells or into nucleus via chloroplast stromules (Krenz et al. 2010, 2012).

Viruses compartmentalize with specific functions *viz.* genome replication or particle assembly like a cell are common. Viral compartments may contain host organelle membranes or are majorly composed of viral proteins. Such viral compartments are called inclusion bodies, viroplasms or viral factories. A virus may form different types of inclusion bodies like CaMV form inclusion bodies of P2 for vector transmission of virus and P6 inclusions for intra- and inter-cellular trafficking of virus in host (Schoelz and Leisner 2017).

CaMV-P6 protein indirectly interacts with cytoskeleton by interacting with chloroplast unusual positioning 1 (CHUP1), a thylakoid membrane-associated motor protein. Thus regulates light absorption through chloroplast repositioning by their movement along actin filaments (Angel et al. 2013). Truncated CHUP1 prevents intra-cellular trafficking while its down regulation delays CaMV infection. Also, P6-CHUP1 interaction is required for aggregation of P6 in inclusion bodies and movement of P6-inclusions to specific intracellular locations e.g. plasmodesmata (Harries et al. 2009; Rodriguez et al. 2014).

Red clover necrotic mosaic virus (RCNMV) has bipartite positive-strand genomic RNA, RCNMV forms punctate structures on cortical ER that contain MP, for intercellular virus trafficking and VRC formation. RCNMV-MP interacts C3 cycle related chloroplast protein, glyceraldehyde 3-phosphate dehydrogenase subunit A (GAPDH-A). Viral RNA localizes GAPDH-Ato cortical VRC and chloroplasts. *Gene silencing* of *GAPDH-A* disables viral-multiplication but intra-cellular viral movement remain unaffected. Also, *GAPDH-A* silencing demonstrated its role in trafficking of RCNMV within host as it is found to hinders localization of MP to cortical VRCs (Kaido et al. 2014).

ToMV movement protein is involved in cell to cell trafficking of virus, symptom development and recognition of resistance gene. ToMV MP shows affinity to interacts with the Rubisco small subunit (RbCS) of *Nicotiana benthamiana. S*ilencing of *RbCS* in susceptible tobacco plants induced necrosis in ToMV inoculated leaves producing local infection while systemic spread and symptoms were delayed. In ToMV resistant tobacco plants *RbCS* silencing *effectively caused local symptoms without systemic spread. So, RbCS* was found crucial for trafficking of virus and in host defence (Zhao et al. 2013).

Expression of TMV CP gene in transgenic tobacco plants is required systemic movement of virus in addition to viral MP (Wisniewski et al. 1990; Reimann-Philipp and Beachy 1993). The interaction of CP of ToMV with interacting

Table 27.4 Chloroplast factors and their viral counterparts involved in cellular trafficking of viruses

Plant virus	Virus components	Chloroplast factors	Biological process	References
Alternanthera mosaic virus (AltMV)	TGB3	Chloroplast membrane	Cell-to-cell movement, long-distance movement, symptom	Lim et al. (2010)
Red clover necrotic mosaic virus (RCNMV)	MP	GAPDH-A	Cell-to-cell movement	Kaido et al. (2014)
Red clover necrotic mosaic virus (RCNMV)	MP	RbCS	Cell-to-cell movement	Zhao et al. (2013)
Red clover necrotic mosaic virus (RCNMV)		IP-L	Long distance movement	Li et al. (2005) and Zhang et al. (2008)
Red clover necrotic mosaic virus (RCNMV)	MP	RbCS	Cell-to-cell movement	Zhao et al. (2013)
Abutilon mosaic virus (AbMV)	MP	cpHSC70-1	Cell-to-cell movement	Krenz et al. (2010, 2012)
Cauliflower mosaic virus (CaMV)	P6	CHUP1	Cell-to-cell movement	Angel et al. (2013)
Cauliflower mosaic virus (CaMV)	P6	CHUP1	Intracellular movement of inclusion body	Harries et al. (2009)

protein-L (IP-L) is essential to establish systemic spread and infection (Li et al. 2005) this protein was screen from tobacco cDNA library by yeast-two-hybrid (Y2H)assay. Zhang et al. (2008) reported that IP-L protein associated with intercellular movement and systemic infection of ToMV is co-localized with ToMV CP in thylakoid membrane of chloroplast suggesting role of this interaction in alteration of chloroplast function and stability resulting in chlorosis symptom.

Hence, aforesaid studies suggest that viruses accomplish their transport via interaction of plant virus components with various cellular factors including chloroplast proteins for regulation of viral movement within host as listed in Table 27.4.

27.4 Role of Chloroplast in Plant Defence System Against Viruses

Symptoms of chlorotic spots, ringspots and necrotic lesions, on the leaves, stems and fruits are produced by virus infection as host's defence responses are evoked in infected cells. Chloroplast is key organelle that provides energy and carbon by photosynthesis. Therefore, during virus infection susceptible plants increase photosynthetic rate to meet high biosynthesis rate in order to fulfil increased

demand of biomolecules that help in establishment of infection. Also, an alternative and opposite plant defence works that tends to cease down photosynthetic rate and other anabolic processes to cut down organic carbon supply to pathogens (Serrano et al. 2016).

Other than photosynthesis chloroplast plays significant roles in crosstalk between plant and external environment, and plant defence. It provides plant defence against biotic and abiotic stresses by producing secondary metabolites including calcium, reactive oxygen species and biosynthesizes plant hormones like SA, JA and ABA that have critical connection with plant immunity (Bobik and Burch-Smith 2015; Kozuleva et al. 2011; Nambara and Marion-Poll 2005; Padmanabhan and Dinesh-Kumar 2010; Seyfferth and Tsuda 2014; Stael et al. 2015; Torres et al. 2006; Wildermuth et al. 2001; Wasternack and Hause 2013). The blockage of SA pathway negatively alters plant resistance to virus infection (Alazem and Lin 2015) while SA and its analogs have positive effect on plant defence and establishment of viral infection is delayed (Radwan et al. 2008; Falcioni et al. 2014). García-Marcos et al. (2013) by gene silencing experiment found that *Coronatine insensitive*$_1$ (*COI*$_1$), associated with JA signalling pathway increases symptoms production and virus accumulation in plant co-infected with PVX and PVY. Thus, chloroplast via intracellular signalling helps to maintain plants cellular homeostasis.

Plant virus use chloroplast as one of the most potent organelle in its infection cycle and forms Chloroplasts form a network of tiny tubes or stomules extended up to nucleus for programmed cell death and defence response that comes in play as chloroplast lack gene silencing mechanism. So, effector triggered defence response of chloroplast works to counter the viral attack. Such effector-triggered response relies on the production of defence responses by directly recognizing the viral virulence factors/effectors or on defence responses produced via detection of plant proteins that have been altered due to viral effector.

For such effector triggered response chloroplast produces network of stromules that are associated with multiplication, spread and host defence against virus (Bhattacharyya and Chakraborty 2018).

Plant cell walls are barrier between cellular and extra-cellular environment. Study of leaf apoplast anti-oxidative system in plum in response to PPV (Diaz-Vivancos et al. 2006) reveals apoplastic protein composition. Apoplastic or secreted extracellular proteins that have host defence response against plant-pathogens including bacteria, fungi and viruses, are called pathogen related proteins. Thus, plant secretome not only help in early recognition and defence against pathogen attack, but also, secretome analyses can improve our insight of plant defence mechanism during plant-pathogen interplays (Yadav et al. 2015a).

Carbohydrate coat on cells is important for biological processes *viz*. viral entry, cell recognition, signal transduction, cell differentiation, cell-cell interactions, bacteria-host interactions, microbial pathogenesis, immunological recognition, fertility and development (Yadav et al. 2015b). Pioneer studies on virus-plant interactions established that accumulation of starch essentially precedes viral symptoms development, suggesting role of carbohydrates in plant defences (Holmes 1931).

Table 27.5 Chloroplast factors and their viral counterparts involved in host defence response

Plant virus	Virus components	Chloroplast factors	References
Plum pox virus (PPV)	CI	PSI-K	Jimenez et al. (2006)
Tobacco vein-mottling virus (TVMV)	CI	PSI-K	Jimenez et al. (2006)
Tobacco mosaic virus (TMV)	RNA helicase domain of replicase	PsbO	Abbink et al. (2002)
	126K replicase	NRIP 1	Caplan et al. (2008)
	126K/183K replicase	ATP synthase-γ subunit (AtpC)	Bhat et al. (2013)
	126K/183K replicase	Rubisco activase (RCA)	Bhat et al. (2013)

Plant virus infections alter carbohydrate metabolism of host via changing rate of biosynthesis and translocation (Gaddam et al. 2012).

Multiplication and spread of virus from diseased plant cells to distant cells and tissues is mediated by host proteins. Hence, comprehensive study of molecular host-virus interactions can provide deep insight in understanding viral replication mechanisms and host defence strategies against viral attack and spread. Also, information on virus-plant interplay enables designing of better virus control strategies. Various chloroplast proteins have been recognised providing defence against viral infection some are listed in Table 27.5.

27.5 Molecular Approaches to Identify Interacting Host Factors During Virus Infection

Viral entry into host cell is dependent on interaction between virus-host molecules i.e., lipids, carbohydrates and proteins. During virus infection cycle, cellular mechanisms of host are dependent on temporally and spatially regulated virus-host protein interactions. Thus, dynamic multiprotein complex formed by virus-host proteins interactions is crucialin binding and uptake of virus into host cell. Genomic or proteomic data of virus-host allow depiction of virus evolution; viral adaptation, interspecies transmissions, and possible emergence of a virulent/pandemic strain (Haagmans et al. 2009). Genomic, proteomic and microarray-based transcriptomic analyses can elucidate gene-interacting networks (Fig. 27.1) of virus-host interplay (Bhattacharjee 2013).

27.5.1 Genomic Approaches to Study Virus-Plant Interplay

Viruses have short replication cycles and produce genomic variants within host to adapt to specific host and to have ability to infect new hosts. Viral evolution occurs

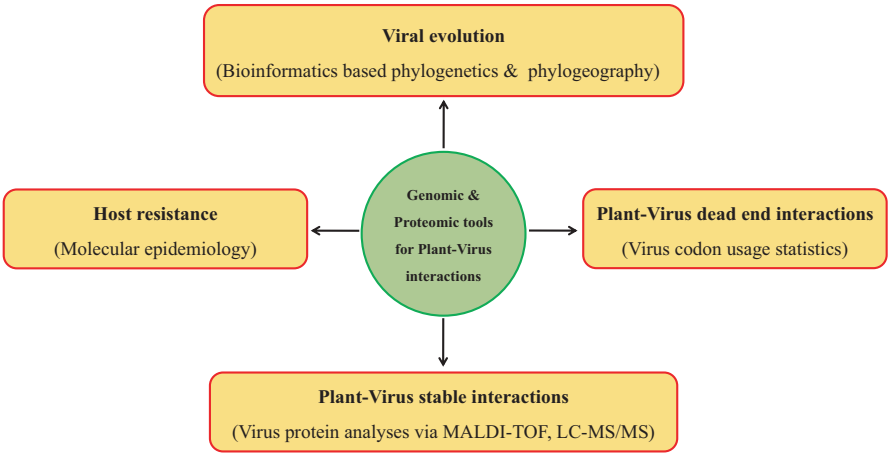

Fig. 27.1 Study of virus-plant interaction using genomic and proteomic tools. (Bhattacharjee 2013)

due to rapid mutation, recombination, and re-assortments within viral genomes and combinations of these processes lead to complex and phenotypically diverse virus populations. Thus, emergence of viral genomic variants from circulating pool within a host population and re-emergence of old ones pose serious threat to agriculture (Bhattacharjee 2013). Genomic techniques like next generation sequencing, rapid genome and transcriptome analyses, methods for evaluation of small-RNAs and their combination with proteomics and metabolomics provides broad understanding of virus-vector and virus-plant interactions.

Genomic studies can address virus-virus interactions, intra and inter-host evolutionary trends, genome wide interactions within viral genomes, ecological interference among different viral groups and virus-host adaptation dynamics (Holmes 2007; McHardy and Adams 2009). Progressively increasing data on virus and host genome sequence and gene expression profiling of transcripts add new insights to understand mechanism of virus infection and host defence. Successful viral invasion depends on compatible virus-host interactions and formation of biologically active functional hetero-complexes that are sensitive to even a point mutation that alters specificity. Gene silencing of PsbP, a host protein increases severity of RSV symptom and accumulation in rice and tobacco (Kong et al. 2014). Thus, change of nucleotide sequence is determinant to map such interactions.

Rodrigo et al. (2012) studied virus-plant interaction using transcriptional regulatory network and protein-protein interaction network for comprehensive understanding of mechanism of host defence in *Arabidopsis* with different viruses. They identified differential regulation of genes by viruses and their involvement in biological function and metabolic processes. These genes are involved in host defence and are either over- or under-expressed on viral infection.

Viruses encode very few proteins yet are capable of altering expression profile of host, as viral effectors act as bridging proteins which interact with signal pathways and interfere with various response factors (Di Carli et al. 2012).

Study of plant virus diversity involves methods *viz*. enrichment of viron or viral genome, extraction of total nucleic acid, high throughput sequencing and bioinformatics. Almost all of these methods have certain limitations but combination of methods can be of great deal for disease management. Metagenomics offers convenient, cost-effective and unique tool to elucidate virus-plant interactions (Stobbe and Roossinck 2014). Thus, metagenomics-based studies of plant viruses enable crop protection and food security (Roossinck et al. 2015).

27.5.2 Proteomic Approaches to Study Virus-Plant Interplay

Plant viruses hijack and use host factors including its proteins, membranes and metabolites for viral-replication and systemic spread. Proteomic analyses of host organelles provides extensive spatial reorganization of host-factors during dynamic virus infection (Gudleski-O'Regan et al. 2012; Horner et al. 2015; Jean Beltran et al. 2016; Varjak et al. 2013), that helps understand molecular mechanism of virus infection.

Being global and specialized method proteomic approach are privileged over genetic, molecular, cellular and biochemical studies, which help to understand virus-plant interplay and identify host-proteins associated with virus infection. Ultimately understanding of virus-plant interactions allows development of new strategies for disease control.

Proteomic approaches allow evaluation of differential protein expression in infected and non-infected cells and identification of virus-host interaction by protein arrays or Y2H assays. Whole proteome-wide approaches identify only host-proteins and not the molecular mechanism during viral infections. Hence, for functional characterization and mechanism prediction proteomics is required to be clubbed with approaches *viz*. genetics, biochemistry, cell biology, etc. Proteomic-based approaches use techniques *viz*. integrative mass spectrometry (MS), antibody-based affinity purification of protein complexes, cross-linking and protein arrays, for elucidation of complex networks proteins allowing virus-host crosstalk during infection. MS-based proteomics and high-throughput genomics methods together allow precise quantitation of proteins involved in complex formation in virus-plant interactions (Gerold et al. 2016). Hence, study of virus-plant interaction using integrative proteomic approaches provides significant insight into the mechanisms of viral replication, anti-viral host response and host defence. Comparative account on various proteomic technologies is listed in Table 27.6.

Sensitive MS-based proteomic methods help in detection and quantitation of variety of proteins from various biological samples including plant viruses. MS, in conjunction with conventional/modern methods enables localization of viral-proteins in host subcellular compartments (Table 27.7).

Tandem affinity purification based MS approach has identifies three host proteins in *Arabidopsis thaliana viz.* heat shock cognate 70-3 (AtHsc70-3), poly(A)-binding (PABP) proteins, and translation elongation factor eEF1A that interact with RdRp of TuMV (Dufresne et al. 2008; Thivierge et al. 2008).

2DE and MS analyses composition of viral-protein complexes and identifies interacting host and viral proteins. Study of purified solubilized active CNV-replicase complex on 2-DE and MS identified two viral and three host proteins *viz*. Heat shock protein 70 (Hsp70), GAPDH, and pyruvate decarboxylase. Additionally 3–7 host proteins strongly interacting with CNV-replicase complex were screened by 2-DE, out of which Cdc34p and Tef1/2p were two were identified using protein array and co-immunoprecipitation (Li et al. 2008, 2009).

ICAT, an adaptation of MS involves labelling of two protein samples i.e., host and virus, with isotopically light and heavy ICAT-reagents that react with thiol group of cysteine residues in proteins. Differently labelled samples are combined, trypsinized and analysed by LC-MSto quantify relative abundance of each protein. It detects low-abundant proteins with more sensitivity than DIGE (Maxwell and Frappier 2007).

DIGE analyses of differential proteomes of resistant and susceptible hosts on SCMV-infection identified 17 new virus-responsive proteins and 7 new proteins without assigned functions that may be associated with new biological functions and significant roles in virus-plant interplay (Wu et al. 2013).

Table 27.6 Comparison of various proteomic technologies used to study virus-plant interactions (Di Carli et al. 2012)

Proteomic technologies		Techniques	Advantages	Limitations
Gel based methods	**2-DE** (two-dimensional electrophoresis)	Electrophoresis (IEF/ SDS-PAGE)	Detects protein modification Cost effective Well established method	No detection of highly basic or acidic/ hydrophobic/ excessively large or small/low expressed proteins Low reproducibility
	2D-DIGE (two-dimensional difference in gel electrophoresis)	Electrophoresis(IEF/ SDS-PAGE)	Detection of protein modification Number of gels reduced compared to 2-DE Reproducible High sensitivity High quality of quantitative analysis	No detection of highly basic or acidic/ hydrophobic/ excessively large or small proteins Expensive dedicated instrumentation and reagents

(continued)

Table 27.6 (continued)

Proteomic technologies		Techniques	Advantages	Limitations
Non-gel based methods	**MuDPIT** (multidimensional protein identification technology)	LC/LC of peptides	High sensitivity than 2DE/ 2DE-DIGE Good detection of hydrophobic proteins Moderate detection of low abundant proteins No pre- or post-separation labelling	No quantitative data High level of MS skills needed Complicated data collection
	Proteome lab PF2D (protein fractionation 2-dimensions)	LC/LC of proteins	Large amount of protein samples Liquid phase of collected fraction Rapid	Low reproducibility Expensive operating procedure
	ICAT (isotope coded affinity tags)	LC of peptides	Detection and quantitation of non-abundant membrane proteins Rapid	No detection of proteins lacking cysteine No detection of protein modification High level of MS skills needed Complicated data collection Only two treatment compared in each experiment
	iTRAQ (isobaric tag for relative and absolute quantitation)	LC of peptides	Quantification of non-abundant proteins/ complex samples Four multiplex labelling method Rapid	Experimental variation due to the lengthy sample processing

Chavez et al. (2012) used cross-linking based MS approach for CP and read through protein of PLRV with diverse biological functions including virus-vector and virus-plant interactions.

Table 27.7 MS based proteomic approaches and their applications in plant virology

MS based proteomic approaches	Virus	Applications	References
Tandem affinity purification based MS approach	*Turnip mosaic virus* (TuMV)	Protein–protein interactions	Thivierge et al. (2008) and Dufresne et al. (2008)
Co-immunoprecipitation based MS approach	*Cucumber necrosis virus* (CNV)	Binding partners of target protein	Li et al. (2008, 2009)
Isobaric tag for relative and absolute quantitation (iTRAQ)	*Cucumber mosaic virus* strain M (M-CMV)	Mapping differently expressed proteins in host proteome upon virus infection	Lei et al. (2018)
Difference gel electrophoresis (DIGE)	*Sugarcane mosaic virus* (SCMV)	Differential proteome analysis	Wu et al. (2013)
Cross-linking based MS approach	*Potato leafroll virus* (PLRV)	Investigating protein-nucleotides binding sites	Chavez et al. (2012)

Hydrogen/deuterium exchange (HDX)-MS approach, an integration of HDX with high resolution MS provides structural information *viz*. protein-nucleic acid bindings, protein-protein cross-talks, and protein maturation rearrangements. HDX-MS thus comprehensively details conformational dynamics of host-viral factors under various biological conditions (Lisal et al. 2005; Zheng et al. 2012).

Y2H detects protein-protein interactions in living cells like plant virus-vector (Liu et al. 2015) and virus-plant interactions. Y2H assay has established the necessity of MP and CP for cell-to-cell movement of *Sesbania mosaic virus* (Chowdhury and Savithri 2011). Tu et al. (2015), studied interaction of PVY-HC-Pro with chloroplast factor ATP synthase $NtCF_1\beta$-subunit using GST pull-down, co-immunoprecipitation and Y2H assays. Crosstalk among cytoplasmic inclusion protein of PPV with chloroplast photosystem I, PSI-K protein in *N. benthamiana* was established by Y2H (Jiménez et al. 2006). Accumulation of a non-structural disease-specific protein (SP) of RSV in rice and tobacco is determinant of severity of symptoms. Interaction between RSV-SP and host proteins PsbP, a 23-kDa oxygen-evolving complex protein, was established by Y2H, GST pull-down and bimolecular fluorescence complementation assays (Kong et al. 2014). Plants being autotropic synthesize amino acids and exhibit lower efficiency for exogenously supplied labelled amino acids which making stable isotope labelling of amino acids in cell culture (SILAC), for plant proteome analyses (Gruhler et al. 2005).

Comparative proteomic studies have been used to understand plant and virus interactions are listed in Table 27.8.

Table 27.8 Comparative proteomics analyses for virus-plant interactions (Di Carli et al. 2012)

Virus	Plant host	Methodology used	No. of differentially expressed host proteins	Classes of plant proteins involved in interaction	Viral counterparts	Type of virus-plant interaction	References
BNYVV (*Beet necrotic yellow vein virus*)	*Beta vulgaris*	Multidimensional protein fractionation system (ProteomeLab PF2D), MALDI-TOF/TOF	10 resistant genotype, 17 susceptible genotype, 21 in both genotypes	–	–	Incompatible, compatible	Larson et al. (2008)
CMV (*Cucumber mosaic virus*)	*Solanum lycopersicum*	DIGE/nLC–ESI–IT-MS/MS	50	Energy metabolism, Defence-stress related	CP	Compatible	Di Carli et al. (2010)
	Cucumis melo	Phloem-SAP pre-fractionation, 2-DE/MALDI-TOF	14	Defence-stress related, Energy metabolism	–	–	Malter and Wolf (2011)
CTV (*Citrus tristeza virus*), CSDaV (*Citrus sudden death-associated virus*)	*Citrus*	2-DE/MALDI-TOF/TOF	–	Defence proteins	–	Compatible, incompatible	Cantu et al. (2008)
GLRaV-1 (*Grapevine leafroll associatedvirus-1*); GVA (*Grapevine virus* A); RSPaV (*Rupestris stem pitting associated virus*)	*Vitis vinifera*	2-DE/MALDI-TOF/TOF	12 (pulp), 7 (skin)	Oxidative stress (skin), Cell structure metabolism (pulp), energy metabolism, Protein turnover, Signal transduction	RSPaV CP	Compatible	Giribaldi et al. (2011)

MYMIV (*Mungbean yellow mosaic India virus*)	*Vigna mungo*	2-DE/MALDI-TOF/TOF	29	Stress related, Energy metabolism, Transport & Signal transduction	–	Compatible (SA treatment reduced susceptibility)	Kundu et al. (2011)
PMeV (*Papaya meleira virus*)	*Carica papaya*	2-DE and DIGE/MS	48 (2-DE); 23 (DIGE)	Defence-stress related, Energy-metabolism, Protein turnover	–	Compatible	Rodrigues et al. (2011)
		1-DE-LC–ESI–MS/MS	10	Plant proteases	–	Compatible	Rodrigues et al. (2012)
PMMoV-S (*Pepper mild mottle virus* Spanish strain)	*Nicotiana benthamiana*	Chloroplast proteins pre-fractionation, 2-DE/MS	36	Energy metabolism, Nitrogen metabolism, Chloroplast large ribosomal protein L14	CP	Compatible	Pineda et al. (2010)
		Chloroplast proteins pre-fractionation, 2-DE/immunoassay/N-terminal sequencing	4	PsbP protein of the oxygen evolving complex	–	Compatible	Pérez-Bueno et al. (2004)
PMMoV-S; PMMoV-I (*Pepper mild mottle virus* Italian strain)	*Capsicum chinense*	2-DE/N-terminal amino acid sequencing/MALDI-TOF MS and MS/MS or n-ESI–IT-MS/MS	14	Defence- stress related (different PR protein isoforms accumulation)	CP	Compatible, incompatible	Elvira et al. (2008)

(continued)

Table 27.8 (continued)

Virus	Plant host	Methodology used	No. of differentially expressed host proteins	Classes of plant proteins involved in interaction	Viral counterparts	Type of virus-plant interaction	References
PPV (*Plum pox virus*)	*Pisum sativum*	Chloroplast & Soluble fraction proteins; 2-DE/ MALDI TOF and n-ESI–IT-MS/MS	12 (chloroplast); 17 (soluble fraction)	Energy metabolism	CP	Compatible	Diaz-Vivancos et al. (2008)
	Prunus persica	Apoplastic fraction 2-DE/MALDI-TOF	4	PR	–	Compatible	Diaz-Vivancos et al. (2006)
RBSDV (*Rice black-streaked dwarf virus*)	*Zea mays*	PEG prefractionation-2-DE/MS	91	Defence-stress-related, Energy metabolism, Cell wall formation, Carbon metabolism, Amino acid metabolism	P9-1 (non-structural viral protein)	Compatible	Li et al. (2011)

RYMV (*Rice yellow mottle virus*)	*Oryza sativa*	Gel exclusion chromatography, SDS-PAGE-nano LC–MS/MS	171 (incompatible cultivar); 135 compatible cultivar	Energy metabolism, Cell wall metabolism, Translation and protein synthesis, Defence-stress related, Transport, Transcription	CP	Compatible, partially resistant, incompatible	Brizard et al. (2006)
	Oryza sativa cv. Indica cell suspension	2-DE/MS	19	Defence-stress related, Energy metabolism, Translation-protein turnover	–	Compatible	Ventelon-Debout et al. (2004)
	Oryza sativa cv. Japonica cell suspension	2-DE/MS	13	Defence-stress related, Energy metabolism, Translation-protein turnover	–	Partially resistant	Ventelon-Debout et al. (2004)
SMV (*Soybean mosaic virus*)	*Glycine max*	2-DE/MS	16	Energy metabolism, Cell wall formation, Defence-stress related, translation-protein turnover	–	Partially resistant	Yang et al. (2011)
TMV (*Tobacco mosaic virus*)	*Capsicum annum*	Nuclei protein pre-fractionation, 2DE/MALDI-TOF	6	Stress related, RNA binding proteins, proteasome	–	Incompatible	Lee et al. (2006)
	Solanum lycopersicum	2-DE/MALDI-TOF MS or LC–MS/MS	17	Defence- stress related	CP	Compatible	Casado-Vela et al. (2006)

27.6 Conclusion

Chloroplast, a dynamic organelle is involved in viral-infection, spread, symptom development and host-defence. Virus-plant interplay involves host and viral proteome regulation. Viruses have evolved strategies to localize viral proteins to organelles like chloroplast by altering its functions to ensure escalated access of host cellular machinery for synthesis and assemble viral components. Virus infection usually invokes down-regulation of host genes encoding chloroplast and photosynthesis related proteins. Analyses of host and viral proteomes, by molecular tools help to identify partner proteins of virus-plant interplay. Hence, selection of candidate chloroplast proteins of virus-plant crosstalk could be targeted for genetic manipulation of plants with minimized adverse effects of viral attack on host metabolism and thus, improving crop productivity without compromising disease resistance.

27.7 Challenges

Chloroplast membrane related proteins with other regulatory proteins participate in virus-plant interactions. Identification and functional characterization of endomembrane proteins using available proteomic methods pose a big challenge as membrane proteins are tough-to-manoeuvre, low abundant and poorly soluble; thus difficult-to-extract. Membrane proteins are low abundant as virus infection seizes cellular pathways and alter protein quantities. Study of such proteins by conventional quantitative proteomics might not be easy but, advanced heterologous protein expression technologies can great help (Yadav et al. 2016).

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