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

Subhasis Karmakar, Kutubuddin A. Molla, and Johiruddin Molla

Abstract

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

S. Karmakar (✉)
ICAR-National Rice Research Institute, Cuttack, Odisha, India

K. A. Molla
ICAR-National Rice Research Institute, Cuttack, Odisha, India

Department of Plant Pathology and Environmental Microbiology, The Huck Institute of the Life Sciences, The Pennsylvania State University, University Park, PA, USA

J. Molla
Center of Excellence in Genomics & Systems Biology, The International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Telengana, India

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

Keywords

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

1 Introduction

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

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

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

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

2 Genetic Engineering of Rice for Biotic Stress Resistance

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

2.1 Rice Disease Resistance (*R*) Genes

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

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

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

Table 1 Summary of the cloned blast resistance genes

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

(continued)

Table 1 (continued)

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

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

2.3 Pathogenesis-Related (PR) Proteins

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

Table 2 Summary of bacterial blight resistant genes in rice

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

(continued)

Table 2 (continued)

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

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

2.4 Antimicrobial Proteins

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

2.5 Defense Signaling Genes and Broad-Spectrum Disease Resistance

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

2.6 Reactive Oxygen Species

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

2.7 Microbe-Derived Elicitor Genes

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

2.8 Gene Pyramiding in Rice for Biotic Stress Tolerance

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

3 Genome Editing System

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

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

3.1 Tools Available for Editing Rice Genome

3.1.1 Zinc Finger Nucleases (ZFNs)

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

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

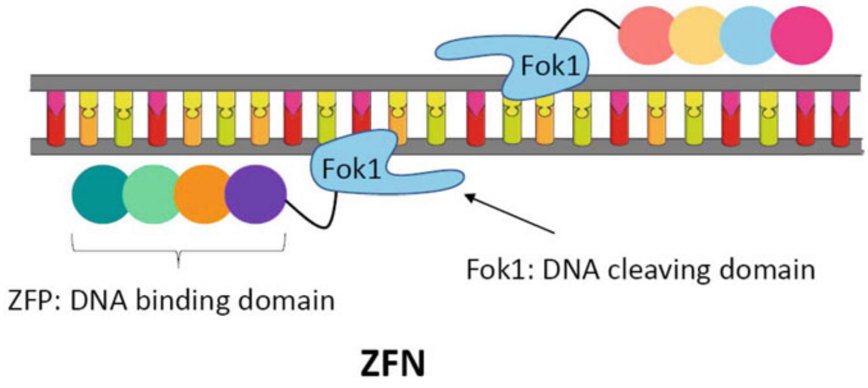

Fig. 1 Basic structure and design of a zinc finger nuclease (ZFN). ZFNs are created by joining a DNA-binding region to the catalytic domain of the nonspecific Fok1 endonuclease. Each zinc finger, illustrated by an individual circle, recognizes 3–4 nucleotides, and, by assembling three or four suitable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

3.1.2 Transcription Activator-Like Effector Nucleases (TALENs)

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

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

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

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

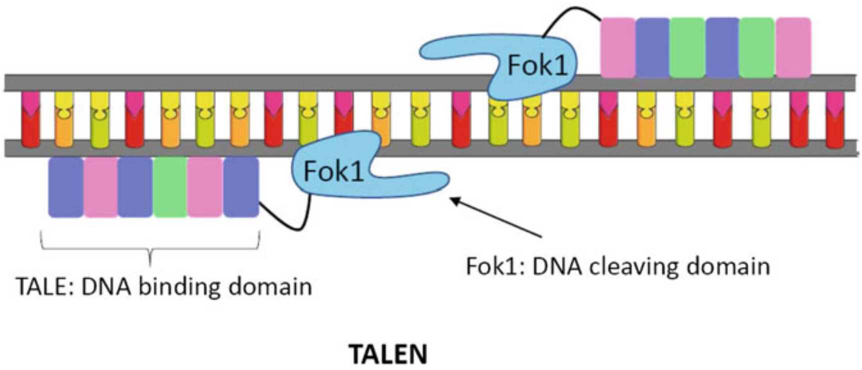

Fig. 2 A scheme for introducing a double-strand breaks using chimeric TALEN proteins. One monomer of the DNA-binding protein domain recognizes one nucleotide of a target DNA sequence. Two amino acid residues in the monomer are responsible for binding. Recognition sites are located on the opposite DNA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

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

3.1.3 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

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

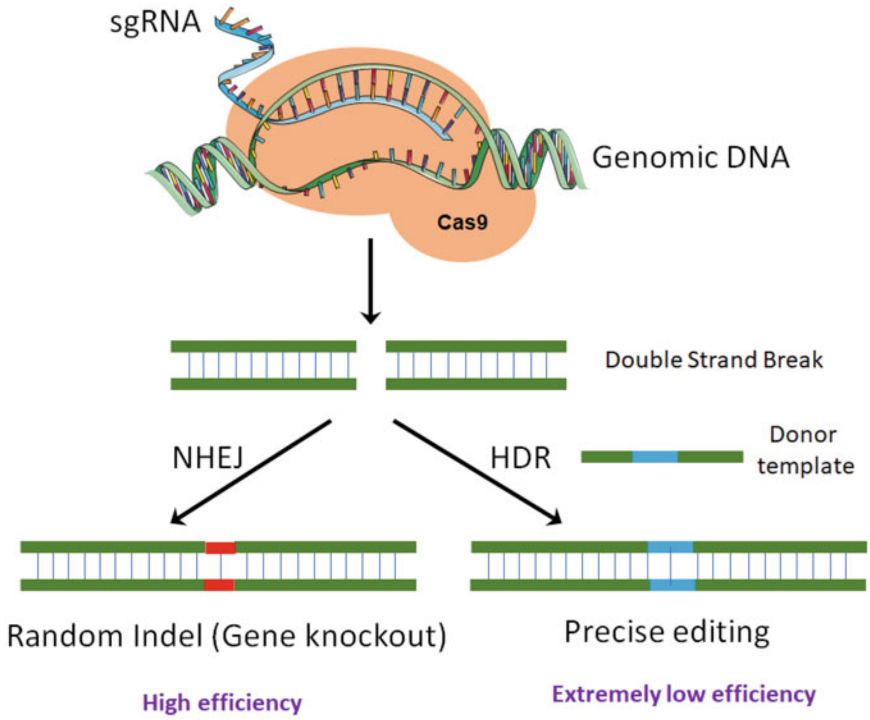

Fig. 3 Schematic depiction of CRISPR/Cas9 genome editing mechanism. sgRNA guides Cas9 to bind and cut specific genomic locus. Once a double-strand break (induced by Cas9) is detected, cellular repair machinery repairs it through either non-homologous end joining (NHEJ) or homology directed repair (HDR)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

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

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

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

3.2 CRISPR/Cas9 System for Biotic Stress Tolerance in Rice

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

4 Future Prospects

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

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

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

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