Genome-Editing Systems in Rice Improvement

Subjects: Cell & Tissue Engineering
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Food crop production and quality are two major attributes that ensure food security. Rice is one of the major sources of food that feeds half of the world's population. Therefore, to feed about 10 billion people by 2050, there is a need to develop high-yielding grain quality of rice varieties, with greater pace. Although conventional and mutation breeding techniques have played a significant role in the development of desired varieties in the past, due to certain limitations, these techniques cannot fulfill the high demands for food in the present era. However, rice production and grain quality can be improved by employing new breeding techniques, such as genome editing tools (GETs), with high efficiency. These tools, including clustered, regularly interspaced short palindromic repeats (CRISPR) systems, have revolutionized rice breeding. The protocol of CRISPR/Cas9 systems technology, and its variants, are the most reliable and efficient, and have been established in rice crops. New GETs, such as CRISPR/Cas12, and base editors, have also been applied to rice to improve it. Recombinases and prime editing tools have the potential to make edits more precisely and efficiently.

Keywords: rice ; grain yield ; abiotic stress ; biotic stress ; grain quality ; food security ; CRISPR/Cas systems ; base editing ; prime editing

1. Introduction

Rice (*Oryza sativa* L.) is grown across the globe and consumed by approximately 3 billion people or around 50% of the world population [1][2]. Rice was grown on 162 million hectares and its global production was 755 million tons in 2019 (http://www.fao.org/faostat/en, accessed on 29 June 2021). The world population may rise anywhere from 9.7 to 11 billion in 2050 (https://population.un.org/wpp/, accessed on 29 June 2021); thus, a significant increase in rice yield will be required to feed the growing population. The global demand for rice is estimated to increase by 50% by 2050 [3]. However, climate change is a major limiting factor for crop production and increases in temperature are leading to more frequent and severe drought spells and soil salinization [4]. Rice faces several biotic and abiotic stresses that significantly lower its production. Approximately 3000 L of water is needed to produce 1 kg of rice, while it is a drought susceptible species due to the thin cuticle wax and small root systems, drought can cause up to 100% yield losses [5]. Similarly, soil salinity could reduce 50% of global rice production [6] and cold stress also threatens rice production and quality [1]. Therefore, an increase in rice production and improvement of its grain quality are essential for healthy and sustainable life in the future [2]. An increase in rice yield and development of stress resilient rice plants are essential for global food security. Moreover, the improvement in grain quality parameters enhances consumer demand and commercial value of rice varieties [7]. Earlier, rice grain quality, climate resilience, disease resistance, and yield have improved via conventional breeding approaches (mutagenesis and hybridization). However, these techniques are time-consuming, tedious, require large mutant screens, and are prone to human biases [4]. Therefore, more powerful, precise, fast, and robust crop improvement approaches, such as genome editing, will be required to meet the rice demand by the ever-growing world population (**Figure 1**).

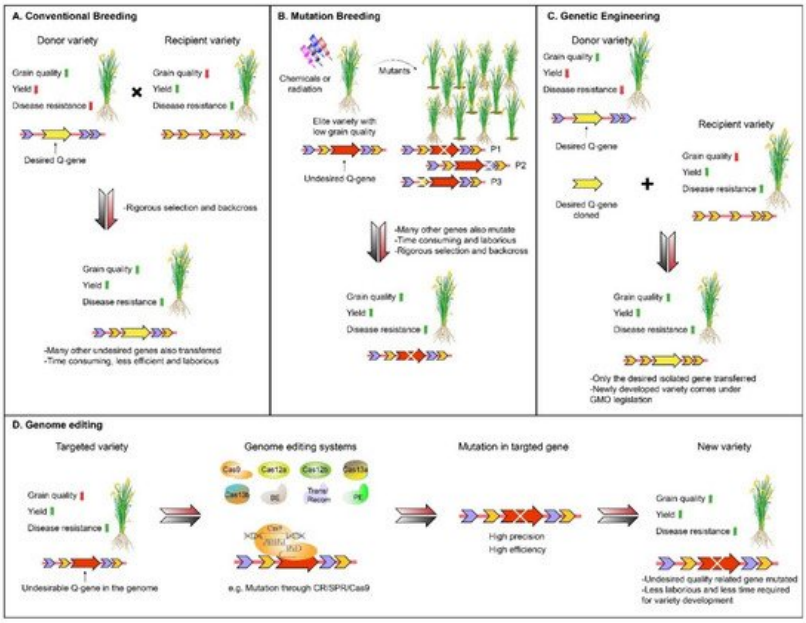

Figure 1. Overview of plant breeding approaches for developing rice varieties with improved yield, stress tolerance and grain quality. (**A**) Conventional breeding for crop improvement, e.g., rice grain quality improvement. (**B**) Mutational breeding approach for rice grain quality improvement. (**C**) Genetic engineering for incorporating the desired gene in popular rice variety. (**D**) Application of gene editing tools for rice plant improvement, e.g., targeting rice grain quality sensitive genes via CRISPR/Cas systems. Upward green arrow shows improved or high grain quality/yield/disease resistance in the plant. Downward red arrow shows deprived or low grain quality/yield/disease resistance in the plant. Q-gene, Quality gene; Cas9, CRISPR-associated protein 9; Cas12a, CRISPR-associated protein 12a; Cas12b, CRISPR-associated protein 12b; Cas13a, CRISPR-associated protein 13a; Cas13b, CRISPR-associated protein 13b; BE, Base Editors; Trans/Recom, Transposases or Recombinases; PE, Prime Editors.

Conventional breeding and mutagenesis techniques drag undesirable genes along with the targeted genes, and take a long time; henceforth, they do not fit the requirements (i.e., of rapidly increasing the production and quality parameters to cope with world hunger and malnutrition challenges). Additionally, hybridization is possible between two plants of the same species, limiting the introduction of new genes and traits. Powerful genome editing technologies (GETs) tackle these limitations of conventional mutational breeding and are capable of transferring a desired trait in any plant species in a short time (**Figure 1**) and, thus, have great potential for speeding up the breeding programs. However, detailed information about the gene sequence, structure, gene function, novel genes and quantitative trait loci (QTL) responsible for traits of interest is vital for application of GETs [8]. GETs modify a specific gene of the desired trait by cutting DNA via target-specific nucleases; thus, the breeding processes are swift. Site-specific endonucleases (SSE), i.e., zinc finger nucleases, transcription activator-like effector nucleases [9], have been introduced in the last decade and are widely used as gene-editing tools.

Recent advancements in GETs involve the development of a clustered, regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system. There are multiple Cas proteins, such as Cas8, Cas9, Cas12a, or Cpf1 (CRISPR from *Prevotella* and *Francisella1*); variants including *F. novicida U112* (FnCpf1) and *Lachnospiraceae bacterium ND2006* LbCpf1); Cas12b, Cas13a, Cas13b, modified forms, i.e., catalytically dead or endonuclease deficient Cas9 (dCas9), nickase Cas9 (nCas9); and orthologues of Cas9, such as *Streptococcus pyogenes* Cas9 (SpCas9), *Staphylococcus aureus* Cas9 (SaCas9), *Streptococcus thermophilus* Cas9 (StCas9), *Neisseria meningitides* Ca9 (NmCas9), *Campylobacter jejuni* Cas9 (CjCas9), etc. [10][11]. They have been used for genome editing through CRISPR technology to improve multiple traits in plants. Among these, the CRISPR/Cas9 system is the most adopted, easier, promising, reliable, and efficient one used for improving yield, stress resilience, herbicide resistance, and end–use quality in several models and crop plants, such as *Arabidopsis thaliana*, *Nicotiana benthamiana*, *Physcomitrella patens*, *Camelina sativa*, barley, corn/maize, citrus, cucumber, soybean, tobacco, tomato, wheat, and rice [12]. Recently, CRISPR-Cas12a and CRISPR-Cas13 systems, which target DNA and RNA, respectively, have been introduced to overcome the limitations of Cas9, owing to their reliability [13][14]. Furthermore, a new technique named base editing (BE), aiming to improve editing technologies by enhancing their proficiency and accuracy, has been introduced and being applied in plant biology [15][16][17][18]. In addition, recombinases and the discovery of prime editing (PE) technology are also used to improve the competence of the genome editing system [19][20][21][22][23]. A detailed comparison of pre-CRISPR GETs, different Cas protein orthologues, prime, and BE technologies have been illustrated in **Figure 2**. Keeping in view with the rapid development in the field of genome editing in general and CRISPR technologies in particular, we discuss

the advancements made in CRISPR/Cas9, modified Cas proteins, base and prime editing systems, with the passage of time, and their applications, to improve rice grain yield, tolerance to abiotic stresses, disease resistance, herbicide resistance, and end-use quality. Due to the direct consumption of rice grain by human beings, the ethics and regulatory aspects of genetically modified rice plants via GETs are discussed. We also focus on the limitations and future prospects of GETs to improve rice for the above-mentioned traits.

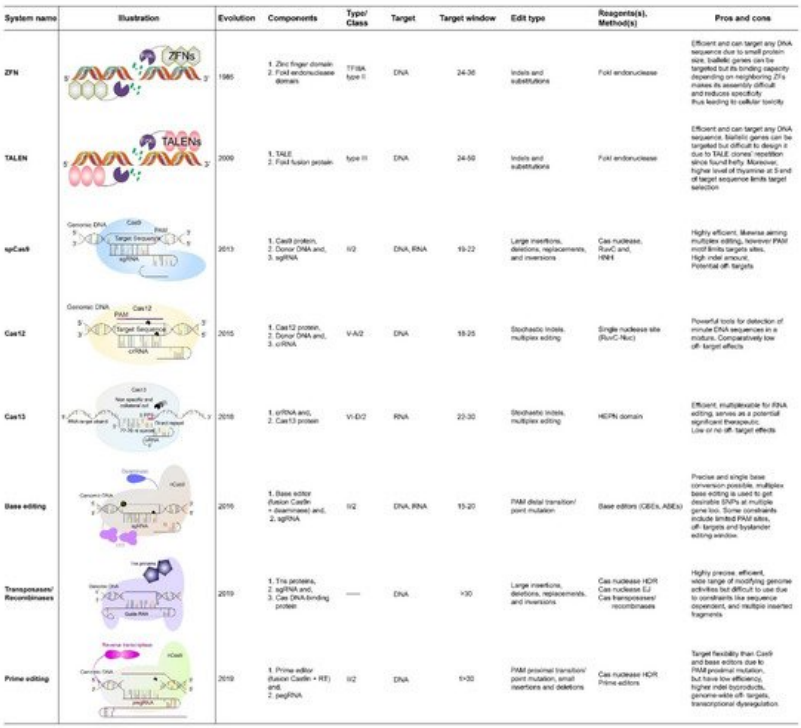

Figure 2. Comparison between different components of various genome-editing tools. The figure presents the models of different genome editing systems, such as ZFN (zinc-finger nucleases), TALEN (transcription activator-like effector nucleases), spCas9 (streptococcus pyogenes CRISPR-associated protein 9), Cas12, Cas13, Base editing, Transposases or recombinases, and prime editing, their evolution, components, class or type of editing system, target nucleic acid, length of target sequence, type of mutations, reagents and methods, and their advantages and disadvantages. PAM, protospacer adjacent motif; sgRNA, single guide RNA; crRNA, CRISPR RNA; Tns Proteins, Transposases proteins; pegRNA, prime editing guide RNA; RT, Reverse Transcription; DNA, Deoxyribonucleic acid; RNA, Ribonucleic acid; RuvC, an endonuclease domain named for an *Escherichia coli* protein involved in DNA repair; HNH, an endonuclease domain named for characteristic histidine and asparagine residues; HDR, homology-directed repair; indel, insertion and/or deletion; CBEs, cytosine base editors; ABEs, adenine base editors.

2. CRISPR/Cas9 Based Rice Crop Improvement

In the CRISPR/Cas9 genome editing system, Cas9 nuclease introduces double strand breaks (DSBs) in DNA at the sgRNA target site. These DSBs are repaired by the non-homologous end joining (NHEJ) pathway that results in insertion or deletions (indels) at the target site, thus knocking out the targeted gene [12] (**Figure 2**). The CRISPR/Cas9 system is the most prevalent GET that has been used to improve several agronomic traits of rice, such as grain yield, abiotic stress tolerance, disease resistance, herbicide resistance, in addition to rice grain quality (**Figure 3**). Classical breeding requires selection of progenies for 6–7 years to obtain the desired level of homozygosity, while the CRISPR/Cas9 system delivers it within a year, making it a powerful plant breeding tool [12]. Herein, we discussed the applications of CRISPR/Cas9 for rice crop improvement.

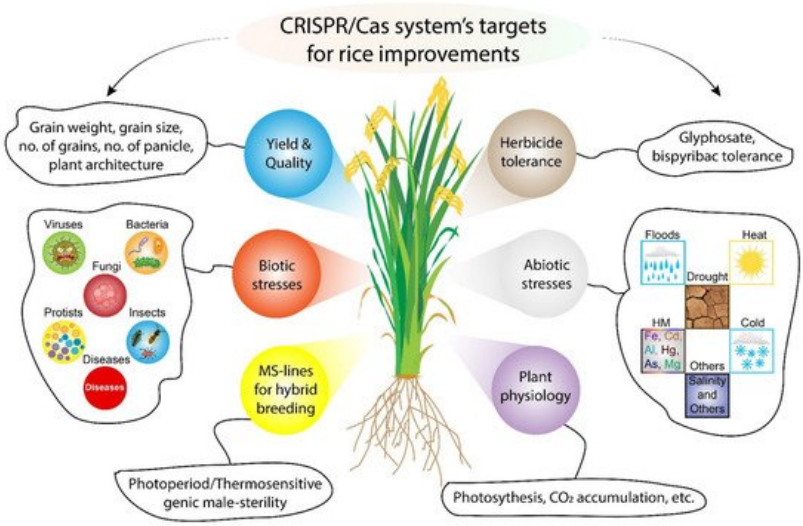

Figure 3. Potential targets of CRISPR/Cas systems for rice crop improvement. Rice can be improved by targeting any potential negative regulator of yield, quality, biotic and abiotic stress tolerance, and plant physiology. Male sterile (MS) lines can be developed for hybrid development by targeting potential genes such as *Thermo-sensitive Male Sterility 5* gene. HM, Heavy Metal; Fe, Iron, Cd, Cadmium; Al, Aluminum; Hg, Mercury; As, Arsenic; Mg, Magnesium.

2.1. CRISPR/Cas9 for Improving Grain Yield of Rice

To date, three distinct strategies have been utilized for improving grain yield of rice by using CRISPR/Cas-based systems:

2.1.1. Improving the Plant Architecture

During the 1960s, manipulation of plant height genes in rice and wheat significantly decreased the plant height, resulting in improved lodging resistance and fertilizer responsiveness that led to considerable increase in grain yield. This is one of the most significant crop improvement events, known as 'the green revolution' [24]. Therefore, altering of rice plant architecture through identification of quantitative trait loci (QTL) and transgene transfer has been a prime breeding target for years [25][26][27]. With the additional benefits of being a robust and transgene-free system, CRISPR/Cas9 has been successfully used to alter the plant architecture by editing/knockout of genes/QTL coding for plant height and number of tillers i.e., *semi dwarf 1* (*SD1*) [28], *STRONG CULM3/TEOSINTE BRANCH1/FINE CULM1 (SCM3/OsTB1/FC1)* [29], *Gibberellin-20 oxidase-2* (*OsGA20ox2*) and *SD1* [30].

CRISPR/Cas9-based mutations in two targets of the first codon of *OsGA20ox2* reduced the flag leaf length, gibberellins level, and plant height (22.2% reduction), and increased the grain yield by 6.0%. Additionally, *OsGA20ox2*, *fructose-bisphosphate aldolase 1*, *glyceraldehyde-3-phosphate dehydrogenase*, *S-adenosyl methionine synthetase 1*, and *putative ATP synthase* proteins were downregulated in semi-dwarf mutants [30]. Similarly, CRISPR mediated *SD1* mutants had resistance to lodging, semi-dwarf plant height, and increased grain yield [28], while *OsTB1/FC1* mutants showed increase in the number of tillers [29]. In another study, CRISPR/Cas9 mediated targeting of *Ideal Plant Architecture 1* (*IPA1*) QTL resulted in reduced plant height and increase in number of tillers and, thus, increased the grain yield [31]. Similarly, targeting of rice *fruit weight 4*, *OsFWL4* [32] with sgRNA/Cas9 led to increases in flag leaf area, grain length, number of tillers, and grain yield.

2.1.2. Improving the Panicle Architecture

Panicle morphology or architecture related traits, such as panicle length, panicle weight, panicle density, panicle orientation (erect or droopy), number of grains per panicle, grain weight, length, and size are the key factors that determine the final grain yield of rice [25][26][27] and, thus, have been a crucial target for high yielding rice. Several genes and/or QTL for panicle architecture traits has been targeted by the CRISPR/Cas9 system to improve the grain yield of rice (**Table 1**). For example, sgRNA/Cas9 mediated multiplexed editing of three panicle architecture regions, i.e., *DENSE AN D ERECT PANICLE* (*DEP1*), *Grain Size 3* (*GS3*), *Grain number 1a* (Gn1a), and a plant architecture QTL, *Ideal Plant Architecture1* (*IPA1*) resulted in improved panicle and plant architecture traits. The mutants had erect panicle, improved grain size, and number of grains, more/less number of tillers and reduced plant height, leading to improved grain yield [31]. Similarly, editing of panicle architecture genes, *PIN family of auxin efflux carrier-like gene 5b* (*OsPIN5b)* genes by sgRNA/Cas9 system increased the panicle length in mutants as compared to wild type plants, thus increasing the rice grain yield [33], while multiplexed CRISPR mediated editing of *Gn1a*, *DEP1* and *GS3* increased the number of grains per panicle, panicle architecture, panicle orientation, grain size, and grain yield [34][35]. Additionally, CRISPR based targeting

of *grain width/weight 2*, *5*, *6* and *8* (*GW2*, *GW5*, *GW6*, *and GW8)* and *GS3* [36][37][38][39] resulted in enhanced grain width, weight, size, and grain yield.

Table 1. Key examples of application of CRISPR/Cas9 system for improving rice grain yield and related traits.

Targeted Trait	Targeted Gene/s	Cas9 Promoter/S	sgRNA Promoter/S	Improved Trait/s in Mutants	Ref.
Plant Architecture	*SD1*	2 × 35S pro	gRNA1^{SD1} gRNA3^{SE5}	Grain yield, plant architecture, semi-dwarf plants, resistance to lodging	[28]
	OsGA20ox2	Pubi-H	OsU6a OsU6b	Grain yield, plant architecture, semi-dwarf plants, reduced gibberellins and flag leaf length	[30]
	SCM1/SD1, *SCM3/OsTB1/FC1*, *SCM2/APO1*	2 × 35S pro CaMV	gRNA1, gRNA2, gRNA3, gRNA4, gRNA5, gRNA6	Plant architecture, number of tillers, panicle architecture, larger panicles, stem cross-section area	[29]
	OsFWL4	Maize Ubi1	OsU6	Grain yield, plant architecture, number of tillers, flag leaf area, grain length, number of cells in flag leaf	[32]
	IPA1	Maize Ubi1	U6a	Grain yield, plant architecture, number of tillers, reduced plant height	[31]
	IPA1, *GS3*, *DEP1*, *Gn1a*	Maize Ubi1	U6a	Grain yield, plant architecture, panicle architecture, number of tillers, grain size, dense erect panicles, grain number	[31]
	GS3, *OsGW2*, *Gn1a*	p35S	OsU6 OsU3	Grain yield, grain size, grain weight, number of grains per panicle	[37]
	OsPIN5b, *GS3*	2 × 35S pro Pubi-H	OsU6a	Grain yield, panicle architecture, panicle length, grain size	[33]
Panicle Architecture	*GW2*, *5 and 6*	pUBQ	OsU3, OsU6 TaU3	Grain yield, grain weight	[36]
	OsSPL16/qGW8	2 × 35S pro Pubi	OsU6a	Grain yield, grain weight, grain size	[38]
	Gn1a, *GS3*	2 × 35S pro	U3	Grain yield, panicle architecture, number of grains per panicle, grain size	[35]
	Gn1a, *DEP1*	2 × 35S pro	OsU3	Grain yield, panicle architecture, panicle orientation, number of grains per panicle	[34]
	Cytochrome P450, *OsBADH2*	Pubi-H	U6a U6b U6c U3m	Grain yield, grain size, aroma (2-acetyl-1-pyrroline (2AP) content)	[39]
ABA Signaling Pathway	*PYL1*, *PYL4*, *PYL6*	Maize Ubi1	OsU6 OsU3	Number of grains, grain yield	[40]
	OsPYL9	PubiH	OsU6a OsU6b	Grain yield under normal and limited water availability	[41]

2.2. CRISPR/Cas9 for Abiotic Stress Tolerant Rice

Constant rise in global temperature is causing global warming, or climate change, leading to more frequent drought spells and soil salinization [4], thus threatening crop production. Rice faces several abiotic stresses during its life cycle and drought is the most eminent threat to rice production. This is due to the fact that 3000 L of water is needed to produce 1 kg rice grains, but a shallow root system and a thin cuticle makes it one of the most drought-susceptible plants that could face 100% yield loss [5]. Similarly, for rice plants grown in the highlands of China, Japan, Korea, etc., cold temperatures during reproduction adversely affect the rice grain yield and quality [1]. Additionally, rice is more susceptible to salt stress as compared to other cereals, such as wheat [42]; thus, rice production could be reduced across the globe by 50% [6]. The situation becomes complicated as drought and salinity tolerance are complex traits that are conferred by several genes, proteins, transporter proteins, transcription factors (TFs), ion transporters, microRNAs (miRNAs), hormones, metabolites, and ions [4][43]. Therefore, classical breeding has limited success and power to accumulate these genes in cultivars and development of abiotic stress tolerant plants. Being a powerful tool that can target any gene in any organism, the CRISRP/Cas9 system has been successfully utilized to improve abiotic stress tolerance in corn, rice, tomato, wheat, *Arabidopsis thaliana*, and *Physcomitrella patens* [12][44]. Indeed, several groups have successfully demonstrated the power of CRISPR/Cas9 system for development of climate resilient (drought, salinity, cold, and osmotic stress tolerant) rice (**Table 2**). Their outcomes and CRISPR-based approaches for the purpose are described below.

Table 2. Examples of CRISPR/Cas9 system for enhancing abiotic stress tolerance of rice.

Stress	Edited Gene/S	Cas9 Promoter/S	sgRNA Promoter/S	Improved Traits in Mutants	Ref.
Drought	***OsSAPK2***	**Pubi-H**	**U3**	**Reduced drought, salinity, and osmotic stress tolerance; role of gene in ROS scavenging, stomatal conductance and ABA signaling**	[45]
	OsPYL9	**PubiH**	**OsU6a OsU6b**	**Drought tolerance; grain yield, antioxidant activities, chlorophyll content, ABA accumulation, leaf cuticle wax, survival rate, stomatal conductance, transpiration rate**	[41]
	OsERA1	**Not defined**	**pCAMBIA1300**	**Drought tolerance, stomatal conductance, increased sensitivity to ABA.**	[46]
	OsSRL1*, *OsSRL2	**Pubi-H**	**U6a U6b U6c U3m**	**Improved drought tolerance; Reduced number of stomata, stomatal conductance, transpiration rate and malondialdehyde (MDA) content; Improved panicle number, abscisic acid (ABA) content, catalase (CAT), superoxide dismutase (SOD) and survival rate**	[47]
	DST	**OsUBQ**	**OsU3**	**Drought tolerance, leaf architecture, reduced stomatal density, enhanced leaf water retention**	[48]
	OsmiR535	**UBI 35S pro**	**OsU3 OsU6**	**Drought tolerance, ABA insensitivity, number of lateral roots (73% more), shoot length (30% longer), primary root length**	[49]
Salinity and Osmotic Stress	***OsSAPK2***	**Pubi-H**	**U3**	**Reduced salinity and osmotic stress tolerance, role of gene in ROS scavenging**	[45]
	OsRR22	**2 × 35S pro Pubi-H**	**OsU6a**	**Salinity tolerance, shoot length, shoot fresh and dry weight**	[50]
	DST	**OsUBQ**	**OsU3**	**Salinity tolerance, osmotic tolerance**	[48]
	OsmiR535	**UBI 35S pro**	**OsU3 OsU6**	**Salinity tolerance, osmotic tolerance, shoot length (86.8%), number of lateral roots (514% as compared with line overexpressing MIR535), primary root length (35.8%)**	[49]
Cold Stress	***OsAnn3***	**UBI 35S pro**	**U3**	**Response to cold tolerance**	[51]
	OsMYB30	**2 × 35S pro Pubi-H**	**OsU6a**	**Cold tolerance**	[33]

2.3. CRISPR/Cas9 for Improving Disease Resistance of Rice

Potato blight in Ireland (also known as the Great Famine or the Irish Potato Famine) during 1845–1853, the Great Bengal Famine during 1943, and maize leaf bight in the USA during 1969–1970 are examples of crop failure due to plant diseases. Some of these events resulted in the deaths and migration of millions [24]. Over 800 million people are underfed across the globe due to plant diseases [24]; thus are a threat to food security. Among the various diseases faced by rice, bacterial leaf blight (BLB), caused by a bacteria *Xanthomonas oryzae pv. Oryza*, is one of the most devastating diseases that can reduce the grain yield by 70% (http://www.knowledgebank.irri.org, accessed on 29 June 2021). Similarly, *Magnaporthe oryzae* fungus causes rice blast disease that could result in 30–100% yield loss [52]. Therefore, management of rice diseases is crucial to feed the growing population.

Improving plant disease resistance through classical breeding approaches, such as back crossing breeding, multiline breeding, and stacking of resistance (R) gene/s, is a lengthy and tedious process that consumes years [53][54]. Whereas, targeting of different susceptibility (S) factors and gene/s through the CRISPR/Cas9 system has fast-forwarded the development of broad-spectrum disease resistance within a year [12][55][56][57][58][59]. Undoubtedly, CRISPR/Cas9 technology has been utilized to develop broad-spectrum resistance against several bacteria, fungi, and viruses (**Table 3**). In rice, two distinct CRISPR/Cas9-mediated approaches has been used to improve disease resistance.

Table 3. Examples of CRISPR/Cas9 system for enhancing the disease resistance of rice.

Pathogen	Improved Disease/Pathogen Resistance	Targeted Gene/S	Cas9 Promoter/S	sgRNA Promoter/S	Ref.
Fungi	Rice blast *(Magnaporthe oryzae)*	*OsERF922*	2 × 35S pro Pubi-H	OsU6a	[60]
		OsALB1, *OsRSY1*,	TrpC, TEF1	SNR52, U6–1, U6-2	[61]
		OsPi21	PubiH	OsU6a, OsU3	[62]
		OsPi21	PubiH	OsU6a, OsU6b	[63]
Bacteria	Bacterial leaf blight *(Xanthomonas oryzae pv. Oryzae)*	*OsSWEET14*, *OsSWEET11*	CaMV35S	U6	[64]
		OsSWEET11 or *Os8N3*	35S-p	OsU6a	[65]
		OsXa13/SWEET11	PubiH	OsU6a, OsU3	[62]
		OsSWEET11, *OsSWEET13*, *OsSWEET14*	ZmUbiP	U6	[55]
		OsSWEET11, *OsSWEET14*	35S CaMV	SW11, SW14	[56]
		OsSWEET14	Pubi or P35S	OsU3, OsU6b, OsU6c	[57]
		OsSWEET14	35S, Ubi	OsU3	[58]
		OsXa13/ SWEET11	35S, Ubi	U3, U6a	[66]
Virus	*Rice tungro spherical virus* (*RTSV*)	*eIF4G*	ZmUBI1, CaMV35S	TaU6	[67]

2.4. CRISPR/Cas9 for Herbicide Resistant Rice

Several herbicides, such as ‘Basta’, and glyphosate N-(phosphonomethyl) glycine are used to kill weeds in over 130 countries [68]. It is important that herbicides just kill the weeds and not the crop plants. Traditionally, DNA recombinant technology or a transgenic approach has been utilized extensively to improve herbicide resistance in corn, cotton, and soybean [68][69]. Due to rigorous biosafety checks for genetically modified organisms (GMO) or transgenic plants, CRISPR/Cas9-mediated herbicide resistance has become more popular in recent years [18][70][71][72]. For example, CRISPR/Cas9-mediated knockout of the *Acetolactate Synthase* (*OsALS*) [73] gene conferred resistance against and imazapic (IMP) and imazethapyr (IMT) in mutant rice plants. Similarly, knockout of the *OsALS* gene [72] and multiplexed editing of *OsALS* and *FTIP1e* genes [18] led to increased resistance against bispyribac sodium and imazamox pesticides, respectively. Moreover, a novel CRISPR/Cas9 mediated knock-in/replacement of *5-enolpyruvylshikimate-3-phosphate synthase* (*OsEPSPS*) gene [71] increased the glyphosate resistance in rice.

2.5. CRISPR/Cas9 for Improving Rice Quality Parameters

Rice grain quality depends on the characteristics that could meet consumer demands and preferences. The grain quality parameters of rice include physical appearance, milling quality, cooking, eating, and some nutritional qualities [2]. The application of the CRISPR/Cas9 system for rice grain quality improvement has speeded up rice breeding with desirable traits (**Figure 4**).

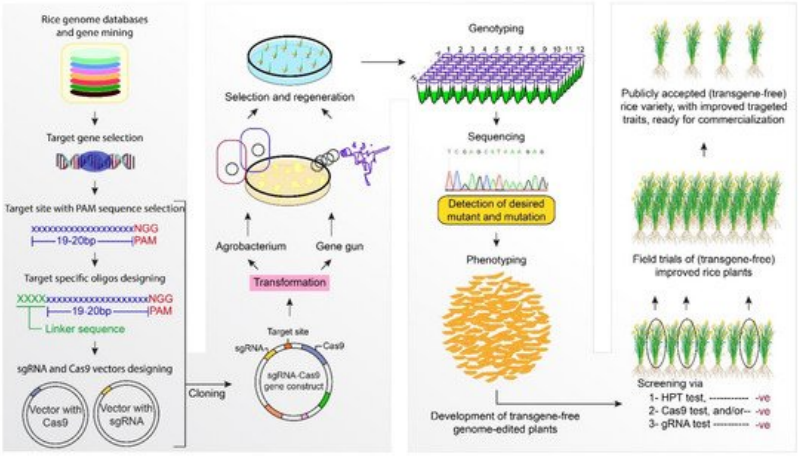

Figure 4. General strategy and stepwise method of development of transgene-free new rice variety with improved desirable traits using genome-editing tools. The process starts with the rice genome databases surfing and gene mining. After selecting the target gene, target site with PAM sequence (NGG) is selected. Then linker sequence (presented in green color) is added on the 5′ of the target site. Then vectors (Cas9 and sgRNA) are constructed and cloned into a new vector that carry the *cauliflower mosaic virus* promoter (CaMV 35S) promoter and vector backbone. Afterwards, the CRISPR/Cas9 construct carrying the target sequence is transformed into rice via a gene gun or the agrobacterium-mediated transformation method. Positive seedlings are screened and phenotyped. Detection of transgene-free plants occurred via HPT (Hygromycin) test, Cas9 or sgRNA test. Afterwards, only transgene-free plants will be further tested in field trials and commercialized.

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