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Review

Applications and Potential of Genome-Editing Systems in Rice Improvement: Current and Future Perspectives

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Abstract: 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. Briefly, in this review, we discuss advancements made in CRISPR systems, base and prime editors, and their applications, to improve rice grain yield, abiotic stress tolerance, grain quality, disease and herbicide resistance, in addition to the regulatory aspects and risks associated with genetically modified rice plants. We also focus on the limitations and future prospects of GETs to improve rice grain quality.

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

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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).

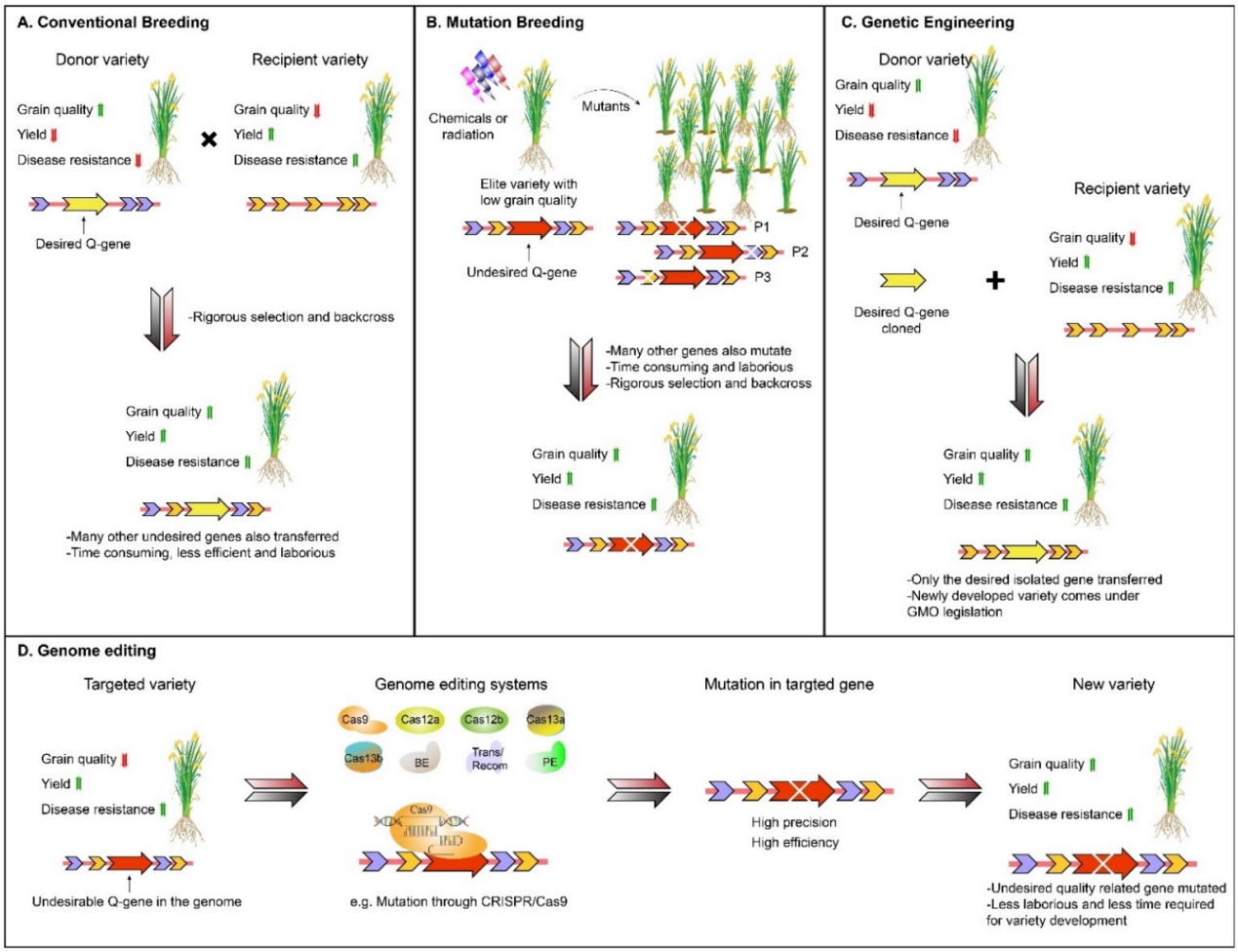


Figure 1. Overview of plant breeding approaches for developing rice varieties with improved yield, stress tolerance and grain quality. (**A**) Conventional breeding for crop improvement, e.g., rice grain quality improvement. (**B**) Mutational breeding approach for rice grain quality improvement. (**C**) 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 [15–18]. In addition, recombinases and the discovery of prime editing (PE) technology are also used to improve the competence of the genome editing system [19–23]. 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.

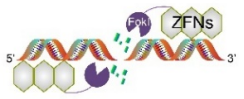
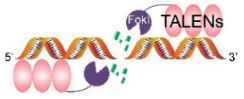
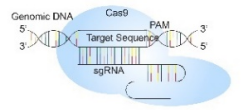
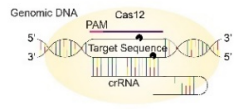
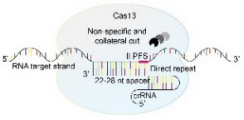
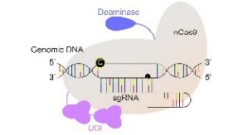
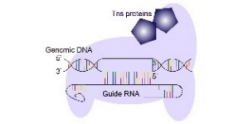
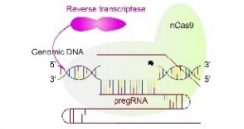
System name	Illustration	Evolution	Components	Type/ Class	Target	Target window	Edit type	Reagents(s), Method(s)	Pros and cons
ZFN		1985	1. Zinc finger domain 2. FokI endonuclease domain	TFIIIA type II	DNA	24-36	Indels and substitutions	FokI endonuclease	Efficient and can target any DNA sequence due to small protein size, biallelic genes can be targeted but its binding capacity depending on neighboring ZFs makes its assembly difficult and reduces specificity thus leading to cellular toxicity
TALEN		2009	1. TALE 2. FokI fusion protein	type III	DNA	24-59	Indels and substitutions	FokI endonuclease	Efficient and can target any DNA sequence, biallelic genes can be targeted but difficult to design it due to TALE clones' repetition since found hefty. Moreover, higher level of thyamine at 5'end of target sequence limits target selection
spCas9		2013	1. Cas9 protein, 2. Donor DNA and, 3. sgRNA	II/2	DNA, RNA	19-22	Large insertions, deletions, replacements, and inversions	Cas nuclease, RuvC and, HNH	Highly efficient, likewise aiming multiplex editing, however PAM motif limits targets sites, High indel amount, Potential off- targets
Cas12		2015	1. Cas12 protein, 2. Donor DNA and, 3. crRNA	V-A/2	DNA	18-25	Stochastic Indels, multiplex editing	Single nuclease site (RuvC-Nuc)	Powerful tools for detection of minute DNA sequences in a mixture. Comparatively low off- target effects
Cas13		2016	1. crRNA and, 2. Cas13 protein	VI-D/2	RNA	22-30	Stochastic Indels, multiplex editing	HEPN domain	Efficient, multiplexable for RNA editing, serves as a potential significant therapeutic. Low or no off- target effects
Base editing		2016	1. Base editor (fusion Cas9n + deaminase) and, 2. sgRNA	II/2	DNA, RNA	15-20	PAM distal transition/ point mutation	Base editors (CBEs, ABEs)	Precise and single base conversion possible, multiplex base editing is used to get desirable SNPs at multiple gene loci. Some constraints include limited PAM sites, off- targets and bystander editing window.
Transposases/ Recombinases		2019	1. Tns proteins, 2. sgRNA and, 3. Cas DNA-binding protein	——	DNA	>30	Large insertions, deletions, replacements, and inversions	Cas nuclease HDR Cas nuclease EJ Cas transposases/ recombinases	Highly precise, efficient, wide range of modifying genome activities but difficult to use due to constraints like sequence dependent, and multiple inserted fragments
Prime editing		2019	1. Prime editor (fusion Cas9n + RT) and, 2. pegRNA	II/2	DNA	1>30	PAM proximal transition/ point mutation, small insertions and deletions	Cas nuclease HDR Prime editors	Target flexibility than Cas9 and base editors due to PAM proximal mutation, but have low efficiency, higher indel byproducts, genome-wide off- targets, transcriptional dysregulation.

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.

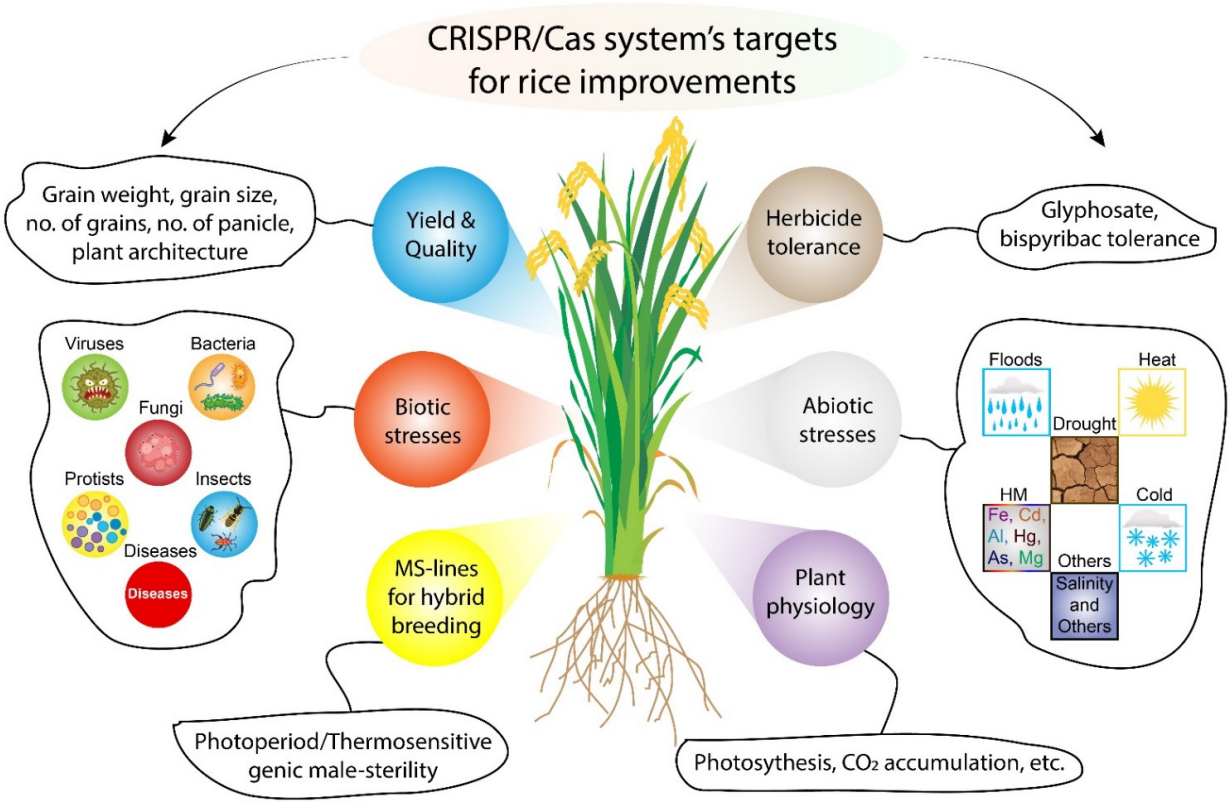


Figure 3. Potential targets of CRISPR/Cas systems for rice crop improvement. Rice can be improved by targeting any potential negative regulator of yield, quality, biotic and abiotic stress tolerance, and plant physiology. Male sterile (MS) lines can be developed for hybrid development by targeting potential 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 [25–27]. 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 [25–27] 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* [36–39] 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]
Panicle Architecture	*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]
	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]

Table 1. *Cont.*

Targeted Trait	Targeted Gene/s	Cas9 Promoter/S	sgRNA Promoter/S	Improved Trait/s in Mutants	Ref.
	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	[41]
	OsPYL9	PubiH	OsU6a OsU6b	Grain yield under normal and limited water availability	[40]

2.1.3. Improving the ABA Signaling Pathway

Abscisic acid (ABA) is an important plant hormone that plays an important role in germination, stress response, plant growth, and development, thus altering the ABA signaling pathway has been an important breeding target [4]. Indeed, CRISPR/Cas9 system based targeting of ABA receptor gene *Pyrabactin Resistance 9* (*OsPYL9)* [40] increased the grain yield and editing of three genes, *PYL1*, *PYL4*, and *PYL6* [41], increased the number of grains by 31%, leading to higher yield in mutants than wild type plants. Thus, this novel approach highlights the potential of manipulation of signaling to increase the grain yield and ensure food security.

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.

2.2.1. Targeting the ABA Signaling Pathway

ABA is the first line of defense against drought stress and its production is one of the first responses to drought stress in plants. Subsequently, plants respond to drought stress through ABA-dependent or independent signaling cascades [4]. CRISPR/Cas9-mediated knockout of *osmotic stress/ABA–activated protein kinase 2*, *OsSAPK2*, revealed that *OsSAPK2* has roles in ABA-mediated seed dormancy, drought, salinity, and osmotic stress tolerance

to rice through ABA signaling, scavenging of reactive oxygen species (ROS), stomatal closure through accumulation of compatible solutes, and upregulating stress-related genes (*OsLEA3*, *OsbZIP23*, *OsRab16b*, *OsRab21*, *OsOREB1*, and slow anion channels, *OsSLAC1* and *OsSLAC7*). The mutant plants were more susceptible to stresses, but this revealed the critical role of *OsSAPK2* in ABA signaling cascade [45]. Similarly, CRISPR/Cas9 system was used to target ABA receptor gene, *OsPYL9* [40], and mutant rice plants had improved drought tolerance due to a reduced number of stomata, stomatal conductance, transpiration rate, and malondialdehyde (MDA) content and enhanced cuticle wax, panicle number, abscisic acid (ABA) content, catalase (CAT), superoxide dismutase (SOD), and survival rate as compared to wild type plants. Drought tolerance in rice was also improved by sgRNA/Cas9 based targeting of *Enhanced Response to ABA1* (*ERA1*) [46] through regulating the stomatal conductance.

2.2.2. Improving the Leaf Morphology

Leaf controls evapotranspiration, an important parameter for drought tolerance. Therefore, altering the leaf morphology is a key strategy to improve grain yield and drought tolerance in plants. CRISPR/Cas9-based multiplexed editing of *SEMI-ROLLED LEAF 1* and *2* (*OsSRL1* and *OsSRL2*) [47] conferred drought tolerance to rice. The mutants had curled leaves, reduced number of stomata, stomatal conductance, transpiration rate, and malondialdehyde (MDA) content, as compared to wild types plants. Additionally, mutants had a higher panicle number, abscisic acid (ABA) content, catalase (CAT), superoxide dismutase (SOD), and survival rate.

2.2.3. Targeting the microRNA and Transcription Factors

The miRNAs and TFs are the key regulators of stress related genes and either up- or-down-regulate the key genes involved in abiotic stress tolerance mechanisms [4,48]. Therefore, knockout of TFs negatively regulating the tolerance related genes is a reliable strategy to increase abiotic stress tolerance in rice. Indeed, CRISPR/Cas9 mediated knockout of the R2R3-type MYB transcription factor, *OsMYB30* [32] increased the grain yield and cold tolerance in mutants as compared to wild type rice plants. Similarly, CRISPR/Cas9 mediated knockout of a zinc finger TF, *drought and salt tolerance* (*DST*) [49], and a miRNA, *OsmiR535* [50], in two independent studies, enhanced drought, salinity, and osmotic stress tolerance in mutants as compared to wild type plants. The abiotic stress tolerance in mutants was conferred by improvement in stomatal conductance, enhanced leaf water retention; leaf, root, and shoot architecture [49,50], as detailed in Table 2. Additionally, sgRNA/Cas9 system based knockout of amino acid B-type response regulator TF, *OsRR22* [51] conferred salinity tolerance in mutants by improving the shoot architecture; thus, highlighting the utility of TF targeting for improving abiotic stress tolerance in rice.

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	[40]
	OsERA1	Not defined	pCAMBIA1300	Drought tolerance, stomatal conductance, increased sensitivity to ABA.	[46]

Table 2. *Cont.*

Stress	Edited Gene/S	Cas9 Promoter/S	sgRNA Promoter/S	Improved Traits in Mutants	Ref.
	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	[49]
	OsmiR535	UBI 35S pro	OsU3 OsU6	Drought tolerance, ABA insensitivity, number of lateral roots (73% more), shoot length (30% longer), primary root length	[50]
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	[51]
	DST	OsUBQ	OsU3	Salinity tolerance, osmotic tolerance	[49]
	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%)	[50]
Cold Stress	*OsAnn3*	UBI 35S pro	U3	Response to cold tolerance	[52]
	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 [53]. 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 [54,55]. 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 [12,56–60]. 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.

2.3.1. Gene Disruption by Targeting the Coding Sequence

This strategy involves the disruption of the coding sequence of S genes and CRISPR/Cas9 is used to create indels in one or multiple nucleotides of exon/s to knockout a particular gene. The most prevalent application of the CRISPR/Cas9 system for disease resistance is to knockout the S genes by targeting the coding sequence (CDS), e.g., gene knockout by targeting the CDS or exons of *Ethylene Response Factor*, *OsERF922* [61], *polyketide synthase*-encoding genes, *OsRSY1*, and *OsALB1* [62], and *PYRICULARIA ORYZAE RESISTANCE 21* (*Pi21*) [63,64] enhanced resistance against rice blast, *M. oryzae*. Similarly, CRISPR mediated targeting of CDS or exons of *Sugar Will Eventually be Exported Transporters* (SWEET) gene

family members, *OsSWEET11* (also called *Os8N3*) [65] and *OsSWEET14* [58] increased the resistance against bacterial leaf blight (BLB) caused by *X oryzae pv. Oryzae* (*XOO*). Moreover, targeting of *eIF4G* [66], a host S gene improved the resistance against or rice tungro disease (RTD) caused by rice tungro spherical virus (RTSV). The disease resistance was observed in terms of less disease symptoms, improved yield, and agronomic performance.

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	[61]
		OsALB1, *OsRSY1*,	TrpC, TEF1	SNR52, U6–1, U6-2	[62]
		OsPi21	PubiH	OsU6a, OsU3	[63]
		OsPi21	PubiH	OsU6a, OsU6b	[64]
Bacteria	Bacterial leaf blight *(Xanthomonas oryzae pv. Oryzae)*	*OsSWEET14*, *OsSWEET11*	CaMV35S	U6	[67]
		OsSWEET11 or *Os8N3*	35S-p	OsU6a	[65]
		OsXa13/SWEET11	PubiH	OsU6a, OsU3	[63]
		OsSWEET11, *OsSWEET13*, *OsSWEET14*	ZmUbiP	U6	[56]
		OsSWEET11, *OsSWEET14*	35S CaMV	SW11, SW14	[57]
		OsSWEET14	Pubi or P35S	OsU3, OsU6b, OsU6c	[58]
		OsSWEET14	35S, Ubi	OsU3	[59]
		OsXa13/ SWEET11	35S, Ubi	U3, U6a	[68]
Virus	*Rice tungro spherical virus* (*RTSV*)	*eIF4G*	ZmUBI1, CaMV35S	TaU6	[66]

2.3.2. Gene Disruption via Promotor Sequence

CRISPR/Cas9 based editing of the promoter sequences of S genes results in blockage of gene expression. The approach is editing of the effector-binding site that makes it impossible for the pathogen-binding site to bind the promotor sequence, thus conferring disease resistance. Certainly, one of the pioneer applications of CRISPR/Cas9 system in plants in 2013 was editing in the promoter region of sugar transporter genes *OsSWEET11* and *OsSWEET14* [67]. Subsequently, CRISPR-mediated targeting of the promoter region of *OsSWEET14* [59], *WEET11/Xa13* [63,68], and multiplex editing of *OsSWEET11 OsSWEET13* and *OsSWEET14* [56,57] genes improved BLB tolerance in rice by avoiding the contact of Xoo with S genes.

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 [69]. 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 [69,70]. 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 [18,48,71,72]. 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).

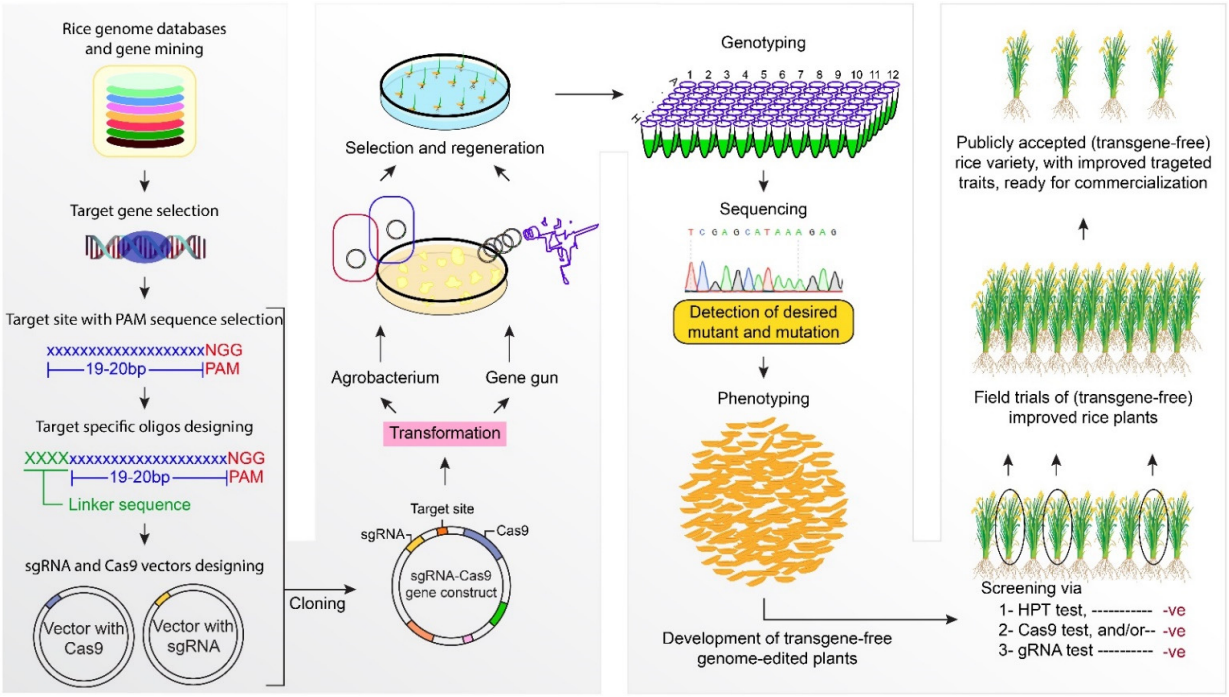


Figure 4. General strategy and stepwise method of development of transgene-free new rice variety with improved desirable traits using genome-editing tools. The process starts with the rice genome databases surfing and gene mining. After selecting the target gene, target site with PAM sequence (NGG) is 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.

2.5.1. Cooking and Eating Quality Traits

Eating and cooking quality (ECQ) traits are at the top of the list due to consumer choices and the economic value of rice [7]. Rice grain endosperm is made up of starch, due to which, grain quality relies on the physicochemical properties of rice endosperm covering amylose content (AC), gel consistency, gelatinization (GT). Genome editing techniques have been used to conduct several successful studies targeting traits for rice grain quality [74]. In the endosperm, AC and GT are regulated by the *waxy or Wx* gene, *disease resistance protein RPM1 (RSR1)* and *soluble starch synthase IIa* (*ALK/SSIIa)*, respectively [75]. The *Wx* gene expression is controlled by a tetratricopeptide domain-containing *FLOURY ENDOSPERM2*, *FLO2* protein, and some other TF like *RSR1*, *granule bound starch synthase (GBSS1)*, *a MYCtranscription factor (OsBP-5)*, *basic helix-loop-helix* (*OsbHLH071*), *Ethylene responsive protein* (*OsEBP-89)*, *basic leucine zipper58 (OsbZIP58)*, and *MADS-box transcription factor 57*, *OsMADS7* [76]. CRISPR/Cas9 was used to introduce a loss-of-function mutation in

the *Wx* gene for reducing AC in widely cultivated japonica cultivars, Xiushui134, and Wuyunjing7 [77]. Such mutations lead to the development of elite cultivars of desired traits without disturbing other traits. Recently, six novel *Wx* alleles have been generated by targeting the *Wxb* promoter to reduce AC in rice grain. These alleles provide a range of AC that can be further used to improve rice quality worldwide according to local environmental conditions and consumer demand [78]. Similarly, ECQ of rice grain has improved by targeting *putative amino acid transporter 6* and *10* (*OsAAP6* and *OsAAP10*) through CRISPR/Cas9 mutation. Mutants developed by knocking out the genes are transgene-free with reduced grain protein content (GPC), AC, and glutelin content, but improved ECQ and starch content in T1 generation, measured by Rapid Visco Analysis [79].

Starch synthesis is regulated by many genes/enzymes, making it hard to alter starch content through conventional breeding. The gene editing of *starch branching enzyme 1* and *2*, *SBEI*, and *SBEII*, resulted in increased AC and resistant starch [80]; thus, offering health benefits. Similarly, CRISPR/Cas9-based mutated *Betaine aldehyde dehydrogenase 2. Badh2* gene (an addition of T base in the first exon of *Badh2* gene, responsible for fragrance) in Zhonghua 11, enhanced the fragrance in rice, a desirable cooking trait [81]. In another study, *isoamylase-type debranching enzyme* (*ISA*), responsible for amylopectin synthesis, was mutated via CRISPR/Cas9, creating *ISA-1* deficient mutants (*isa1*), to improve grain quality with a change in expression of the gene/s associated to starch synthesis, total soluble sugar, and grain weight [82]. Further details about gene functions, cultivar background, Cas9, and sgRNA promotors for improving rice grain quality are provided in Table 4.

2.5.2. Physical Appearance and Milling Quality

Rice grain appearance and color is an important quality trait that determines the rice market acceptability [2]. Many genes, for grain size and shape affecting yield and quality, have been discovered, having the potential to be improved by CRISPR technology (Tables 1 and 4). Chalkiness is an undesirable attribute related to large grain size, resulting in high yield but poor quality, as it negatively affects the grain appearance and milling [83]. The *GS9* gene (*grain shape gene on chromosome 9*) has been modified via CRISPR/Cas9 as *gs9* allele in Nipponbare by improving grain shape and appearance quality, while chalkiness was significantly reduced [84].

Grain length and size are important appearance quality parameters and many rice consumers prefer longer grains. CRISPR/Cas9-based editing of *GS3 (GRAIN SIZE 3)* has been used to increase the grain size and length [31,33]. Recently, CRISPR/Cas9-mediated mutagenesis of three cytochrome P450 including (Os03g0603100, Os03g0568400, and GL3.2) and *OsBADH2* genes led to increase in grain size and fragrance [39]. In another study, CRSISPR based editing of the *OsFWL4* gene significantly increased the rice grain length [32]; thus, increasing the consumer acceptability.

2.5.3. Nutritional Quality Traits

Iron (Fe), zinc (Zn), iodine, vitamin A, folate or vitamin B_9, proteins, fats, and amino acids are essential for human health, and their deficiency could increase the risk of depression, obesity, anemia, type II diabetes, pregnancy related complications, cancers, and cardiovascular disease [2]. Tilling based mutations in DNA demethylase gene, *OsROS1* or *TA2* resulted in enhanced antioxidants, lipids, phenolic, proteins, dietary fiber, vitamins (A, B1, B2, B3, B6, and E), and minerals (calcium, Fe and Zn) in rice grain [85]. However, large populations are required TILLING mutant screens due to low editing efficiency. Therefore, CRSIPR/Cas9 system with high efficiency has been used for the purpose. For example, CRSIRP/Cas9 based knockout of the *Manganese/metal transporter 5* (*OsNramp5)* gene resulted in a more than six-fold reduced accumulation of toxic metal, cadmium (Cd), in rice grain [86], while copper accumulation increased and Zn concentration remained unchanged, as compared to wild type plants. Similarly, CRSIRP based editing of *OsNramp5* produced the similar results [87] as detailed in Table 4. Additionally, CRISPR-Cas9 knocked out of *inositol 1,3,4-trisphosphate 1/6-kinase* (*OsITPK1-6*) led to low phytic acid accumula-

tion in the rice grain and ultimately increased the inorganic phosphorus availability [88]. Moreover, CRISPR mediated knockout of the rice Orange (*OsOr)* gene [89], and insertion of *Calreticulin 1* (*SSU-crtI)* and *Phytoene synthase* (*ZmPsy)* genes [90] resulted in increased β-carotene or provitamin A in rice that must be an useful alternative to brown rice.

Table 4. Key applications of CRISPR/Cas9 system for improving rice grain quality.

Quality Traits	Cultivar Background	Targeted Gene	Gene Function	Cas9 Promoter	sgRNA Promoter	Results	Ref.
Eating andcook-ingquality	Yanggeng-158 Nangeng-9108 Wuyungeng-30	*OsAAP6 OsAAP10*	Amino acid transporter for GPC	CaMV35S	OsU3	Improved eating and cooking quality	[79]
	Zhonghua11, XS134	*OsWaxy*	GBSS (amylose synthesis)	CaMV35S	OsU6	Decrease in amylose content (glutinous rice)	[78]
	XS134 (Japonica)	*OsWaxy*	GBSS (amylose synthesis)	CaMV35S	OsU6	Decrease in amylose content (glutinous rice)	[77]
	Kitaake (Japonica)	*OsBEIand OsBEIIb*	Starch branching enzyme	pCXUN	OsU3	High amylose content	[80]
	Zhonghua11	*ISA1*	Starch (isoamylase-type) debranching enzymes	CaMV 35S	OsU6	Reduced amylose content; increased total soluble sugar	[82]
	Indica	*BADH2*	Betaine aldehyde dehydrogenase (fragrant rice)	OsUbi	OsU6a	Enhanced fragrance	[81]
	Zhonghua 11 (Japonica)	*GS9*, *DEP1*	Grain size, Panicle architecture	OsUbi	OsU6a	Slender grain shape, less chalkiness	[84]
	IR-96	*Cyt P450 homoeologs*, *B OsBADH2*	Grain yield and fragrance	Pubi	OsU6a, OsU6b, OsU6c, OsU3m	Increased grain size, and fragrance	[39]
Physical and appearance quality	Indica (VP4892)	*OsSPL16/GW8*	Grain size	pUbi	OsU6aOsU6b	Increased grain size	[38]
	Zhonghua-11 (Japonica)	*GS3/Gn la*,	Grain size-3/grain number la	OsUbi	OsU6a	Increased grain length	[31]
	Japonica	*GS3*	Grain size-3	2 × 35S Pubi-H	OsU6a	Increased grain size	[33]
	Nipponbare (Japonica)	*GW2/GW5/ TGW6*	Grain weight	OsUbi	OsU3, OsU6, TaU3	Increased grain length and width	[36]
	Japonica	*OsFWL*	Grain Length	Maize Ubi1	OsU6	Increased grain length	[32]
Nutritional Quality	Indica	*OsNramp5*	Cd accumulation	ZmUBI	OsU6a	Low Cd in grains	[87]
	Indica (Huazhan and Longke 638S)	*OsNramp5*	Cd accumulation	Pubi-H	OsU6a	Low Cd in grains	[86]
	Rice Protoplast	*OsOr*	β-carotene synthesis	2 × 35S	OsU6-2	Increased β-carotene (provitamin A) content	[89]
	Kitaake	*SSU-crtI*, *ZmPsy*	β-carotene synthesis			Increased β-carotene (provitamin A) content	[90]

3. Prime Editing and Cas Variants for Rice Crop Improvement

PE is a newly developed system utilizing modified Cas9 protein/s and PE guide RNA (pegRNA) that ensures the cut is made in a single strand instead of both DNA strands in a traditional CRSIRP/Cas9 system, and improves the accuracy of genome editing (Figure 2). In rice, 179 predicted off-target sites were targeted by PE i.e., 12 pegRNAs and a Cas9 variant, nCas9 (H840A) but off-target edits (indels) had quite low frequencies of 0.00~0.23% [91]. This explains the power of PE system for precise, targeted, and accurate genome editing. The PE system can produce 12 kinds of point mutations, including all six possible base pair conversions, base substitutions, insertions, and deletions in rice protoplast [19] that make it a powerful crop improvement system.

Recent research on PE application in rice has been conducted by constructing prime editors Sp-PE2 and Sp-PE3 using Cas9 variant, SpCas9. An expression cassette comprising pegRNA, ZmUbi promoter, OsU6, and an inactive EGFP driven by CaMV35 promoter was

inserted into Sp-PE2, and Sp-PE3 was used to observe mutations. Sp-PE2 has a higher efficiency, showing a strong GFP signal as compared to Sp-PE3. These PE were used to edit *ALS*, and *BERRANT PANICLE ORGANIZATION 1* (*APO1)* genes, thus generating stable mutant lines [23]. Similarly, plant prime editor 3 version 1 (PPE3-V01) with engineered (M-MLV) RT and Cas9H840A were optimized for its application in rice cells. Five target sites in four rice genes (*OsKO2*, *OsDEP1*, *OsDPS*, *OsALS*) confirmed the PE activities and found SNPs and indels at variable frequencies [21]. Another PE system (pPE2) was found to be an efficient system for precise genome editing, targeting different genome sites in rice. The pPE2 system performed better than the pPE3 system for different genomic sites. Moreover, a surrogate pPE2 system in which the *hygromycin phosphotransferase* (*HPT*) reporter gene with an ACG substitution at the start codon (HPT^{-ATG}) was incorporated into prime edited cells, thus easily detecting nucleotide editing, and developed new corridors for flexible editing in rice [92]. Applications of PE systems and Cas protein variants, such as dCas9 and Cas12a/Cpf1 to improve the genome editing efficiencies, plant growth and development, plant architecture, herbicide resistance, etc., are detailed in Table 5.

Table 5. Examples of application of prime editing and Cas proteins variants for rice crop improvement.

Systems	Cultivar Used	Gene Name	Gene Function	Cas9 Promoter	sgRNA Promoter	Results	Ref.
dCas9	Zhonghua 11	*OsGW7* *OsER1*	Grain size and shape, ethylene upregulation	CaMV35S	OsU6a	Multiplex genome editing	[93]
	Rice protoplast	*OsSPL14*, *OsIPA*, *OsGRF1*	Senescence, plant architecture	Ubi		Generate larger deletions	[94]
Cas9 with APOBEC	Rice protoplast	*OsAAT* *OsNRT1.1B* *OsCDC48*	senescence, cell death	Ubi	CaMV	Generate short and larger deletions	[95]
Cas12a/Cpf1 FnCpf1 and CRISPR/ LbCpf1	Nipponbare	*ALS* (Acetolactate synthase)	Synthesis of branched chain amino acids	ZmUBI	OsU6	Loss of *ALS* activity, plant death	[96]
	Rice protoplast	*DEP1*	Dense and erect Panicle	CaMV35S	OsU6	Scattered panicle	[97]
	Rice protoplast	*OsBEL*, *OsPDS* *OsEPSPS* *OsBEL OsRLK*	*Bentazon-sensitive-lethal*, Phytoene Desaturase	CaMV35S	OsU6	Multiplex gene editing (Albino phenotype)	[98]
Prime editors	Rice protoplast	*OsALS* *APO1*	*Acetolactate synthase*, Panicle organization	CaMV35S	OsU6	Resistant to imidazolinone herbicides	[23]
	Nipponbare	*OsPDS1* *OsACC1* *OsWx1*	Herbicide resistance, amylose	Ubi-1 CaMV35S	OsU3	Enhanced herbicide tolerance	[92]
	Rice protoplast	*OsALS*, *OsACC* *OsDEP1*	Nitrogen use efficiency Herbicide tolerance	ZmUbi1 OsUbq	OsU6a OsU3	Nucleotide substitution, herbicide resistance	[20]
	Rice protoplast	*OsALS*, *OsKO2*, *OsDEP1*, *PDS*	Panicle Architecture	ZmUbi1	OsU6 OsU3	Novel prime editing, dense panicle architecture	[21]

4. Base Editing for Rice Crop Improvement

BE is a novel genome editing tool that utilizes CRISPR components to introduce point mutations or single-nucleotide variants (SNVs) in the genome without making DSBs in DNA and without reliance on HDR (Figure 2). Base editors either contain catalytically

impaired Cas nuclease that cannot make DSBs or proteins that manipulate DNA repair machinery to turn on NHEJ instead of HDR [99]. There are two types of base editors, i.e., cytosine base editors (CBEs) and Adenine base editors (ABEs). The Cas9 variants, such as xCas9, SpCas9, and SaCas9, used in the cassette of CBEs increased the mutation frequency of C•G to T•A up to 80% in rice [100]. Recent base editors with Cas9 variants (nSpCas9, nCas9, SaCas9) improved the editing efficiency of ABEs (A•T to G•C conversions) in plants, including rice, for several applications in crop improvement [100,101]. Increased ABE efficiency in two target genes *OsSPL14* and *OsSPL17*, with improved PAM compatibility in rice, expand its application in other crops [100]. Better performance in rice could be evident using ABE and CBE systems, simultaneously [102]. Generating larger and precise deletions was challenging to achieve, but now has been reported using a cassette of Cas9, uracil-DNA-glucosidase (UDG), and a lyase (apurinic/apyrimidinic site). The generation of APOBEC-Cas9 fusion induced deletion system (AFIDs) [95] resulted in large uniform deletions in rice and wheat protoplast that can be used to determine their regulations and protein domains for crop improvement. BE has been used to improve grain yield, grain quality, and herbicide resistance in rice (Table 6).

Table 6. Applications of base editing for rice crop improvement.

Gene Name	Gene Function	Base Editing Tool	PAM	Editing Window (nt)	Target Trait	Ref.
OsCDC48, *OsNRT1.1B* *OsSPL14*	senescence, cell death, plant architecture	pnCas9-PBE	CGG	3 to 9	Yield	[103]
SPL14, *SPL17*, *SPL16*, *SPL18*	Grain weight, size, shape, quality, number	ABE-P1 ABE-P2 ABE-P3 ABE-P4 ABE-P5	GAG CAG CGA GGA AGCG GGCG	3 to 15	Yield	[102]
SLR1, *SPL14*, *SPL16*, *SPL18*, *SPL17*	Grain weight, size, shape, quality, number	ABE-P1 ABE-P2	NNGRRT	4 to 9	Yield	[17]
NRT1.1B	Nitrogen transporter	APOBEC1-XTEN-Cas9(D10A)	AGG GGG	5	High nitrogen use Efficiency	[104]
SBEIIb	Starch branching enzyme	CBE	CCT	5	High amylose	[105]
Wx or GBSSI	Starch synthesis	CBE	CCA CGG	4	High amylose	[106]
OsALS1	ABC transporter	BEMGE	CGG CAG	5–7	Herbicide resistance	[107]
OsALS1	ABC transporter	CBE	CGG CCT	3–5	Herbicide resistance	[108]
OsACC	Herbicide resistance	eABE eBE3	TGG	3 to 9	Herbicide resistance	[109]

4.1. Grain Yield and Related Traits

BE systems, particularly ABE has been used to introduce point mutations in several *Squamosa Promoter Binding Protein-Like* genes, such as *OsSPL14*, *SPL16*, *SPL17*, and *SPL18* [16,102,103], to target plant architecture, grain weight, size, and shape, in order to improve the yield. Similarly, BE has been used to target nitrate transporter 1, *OsNRT1.1B* [103,104] to improve the nitrogen use efficiency of rice plants as an indirect strategy to improve the grain yield of rice.

4.2. Grain Quality

Some gene/s controlling rice grain quality has been modified through BE. For example, an efficient cytosine base editor 3, CBE3, system was applied to alter three target sequences in two rice genes simultaneously, including two targets in *OsBEIIb* (*starch branching enzyme*

2b) named (S3 and S5), and another target site (P2) in rice Phytoene Desaturase (*OsPDS*). This system resulted in desired precise point mutations having a mutation frequency of 19.2%, 10.5%, and 1.0% at S5, S3, and P2 targets, respectively. The resultant mutations in these genes resulted in high amylose contents in rice [105]. Similarly, transgene-free mutants produced by targeting *Wx* gene/GBSS1 protein using a cytidine BE system [106] changed the amino acid sequence and altered amylose contents of rice grain.

4.3. Herbicide Resistance

BE systems have been used to improve the herbicide resistance in rice by targeting *OsALS* and *OsACC* genes [107–109]. Among these, a novel base-editing-mediated gene evolution based breeding strategy was used to target 3–5 nucleotides of *OsALS* gene that conferred resistance against bispyribac-sodium herbicide [107]. Similarly, CBE system was used to introduce missense mutation in two codons of *OsALS* [108]. This novel breeding approach resulted in resistance against five herbicides i.e. bispyribac-sodium, Flucarbazone-sodium, imazapic (IMP), nicosulfuron, and pyroxsulam.

5. Regulatory Aspects and Risks Associated with Genome Editing

Genome editing, predominantly CRISPR technology, certainly has a great potential for revolutionizing plant science as it can create genetic variants like that of natural variants. The main concern for these edited crops is their regulation across the world. The legislation and regulatory framework are evolving in different countries; however, we need to know the recent policies in Europe to adopt new technologies [110]. Genome editing is categorized by site-directed nucleases (SDNs), generating variations in the host genome. TALEN or Cas9 targets a specific site generating a DNA break that is impaired by the plant's natural DNA repair mechanism (Figure 2). SDNs resulted in target site variants causing three types of modifications, such as SDN-1, base-pair changes due to small deletions, or insertions with no foreign DNA. SDN-2 causes a specific change due to homologous recombination using a small DNA template, and the SDN-3 approach is just like SDN-2, but uses a larger DNA segment [111]. A plant having a larger segment of foreign DNA is identified as a transgenic plant. Biosafety legislation of such plants is approved by adaptation of SDN-induced variants in many countries [110]. The EU Court of Justice (ECJ) categorized all genome-edited organisms as GMO.

Other than the EU, New Zealand also considers genome edited plants as GMOs and regulates these mutants under GM biosafety rules. Organisms generated through conventional mutagenesis are exempted because of a safe use history record. ECJ had a negative impact on agricultural innovation, as only 8% of CRISPR patents originate from Europe, while 60% from China and 26% from the USA. In October 2019, the European Union demanded to highlight GETS status, which would have been compiled in April 2021. Future application of genome editing, their ethical and societal issues, and a risk assessment framework will be proposed by the European Food Safety Authority (EFSA). Argentina follows regulatory criteria for gene-edited crops as they classified SDN-3 generated crops under GMOs; however, crops modified by SDN-1 as a non-GMO. Crops generated by SDN-2 do not follow any regulatory criteria [112].

Brazil, Chile, and Columbia also follow the same regulatory criteria as in Argentina. If plants do not have any foreign DNA, they will not be regulated as GMOs. Similar to the USA, Canada is also conscious about the 'novelty' of traits for regulatory assessment. Plants propagated by SDN-1 and SDN-2 have to follow the same assessment or regulation established by the Canadian Food Inspection Agency (CFIA) and Health Canada [113]. Japan employs the same rule (if plants do not have any foreign DNA, they will not be regulated as GMOs), thus they are regulated as conventional crops and can be consumed without any safety evaluation, but this suggestion must still be adopted formally by the Ministry of health [114]. In Australia, the Gene Technology Regulations Review was presented with updated amendments. These amendments classified SDN-2 and SDN-3 developed plants as GMOs, and SDN-1 plants as non-GMOs [115], but still need government approval.

China focuses on processes or techniques used to create new crop varieties and provides huge financial support. No rules for gene-edited crops have been proposed yet but we expect that China will regulate gene-edited crops in a required manner, just like Japan. Recently, France opposed the EU court's decision by not considering GETs under strict GMO biosafety rules. As the largest agricultural producer or the EU, it will consider changing the administrative court's decision on GET regulations (https://www.reuters.com/article/france-agriculture-gmo/france-backs-non-gmo-regulation-for-crop-gene-editing-in-eu-idINL8N2JT4A3 accessed on 29 June 2021). However, in the present era, scientists should collaborate to harness the potential of CRISPR technology, for example, a joint statement in support of agricultural applications supported by 13 countries, including the United States, Canada, Argentina, Australia, Brazil, Colombia, the Dominican Republic, Guatemala, Honduras, Jordan, Paraguay, Uruguay, and Vietnam. This statement is a good sign for overwhelming differences among countries regarding regulatory frameworks, thus empowering innovative agriculture. Though the CRISPR-Cas system is producing transgenic free plants, GMO regulation is acceptable by the scientific societies of some countries only.

6. Limitations and Solutions

GETs have great potential over conventional breeding in developing high-quality rice varieties due to their high efficiency, precision, robustness, and multiplex editing ability. Regardless of GET efficiency (especially the CRISPR-Cas system) and their vast application, they still have some limitations that hamper their usage for crop improvement. Some of these hindrances are:

- Disruption/mutation of the targeted gene may cost some fitness, as it can disturb the pathway of the product or any product or element involved in this pathway. A gene has a linkage with many other genes and regulating pathways. This fitness cost may affect genes regulating plant growth and development, deficiency of essential nutrients leading to visual abnormalities. In order to overcome this limitation, the BE method is applicable, targeting a single nucleotide mutation, escaping disruption of other genes, or targeting promoter to generate alleles [116].
- "Off-target mutations" is another major limitation and significant support for improving the CRISPR system [117]. Unintended or undesired DNA modifications created by deceptive gRNA or a gRNA- independent method or non-specific sites fall under off-target mutations [118]. Possible solutions to cope with off-target mutations and production of transgene-free crops are improving the CRISPR system for precise and reliable editing, or developing an approach to identify off-target mutation. Some bioinformatics tools have been established that can detect off-targets, i.e., Cas-OFFinder (http://www.rgenome.net/cas-offinder/, accessed on 29 June 2021) and CCTop (https://crispr.cos.uniheidelberg.de, accessed on 29 June 2021), and also some systems, such as SELEX, IDLV capture, Guide-seq, HTGTS, BLESS, Digenome-seq [119] and DISCOVER-seq [120]. Still, researchers must use these tools according to the requirements due to their specific pros and cons. In contrast, Cas9 proteins have been modified for improved target specificity, including eSpCas9 [121], HF-Cas9 [122], HypaCas9 [123], and Sniper Cas9 [124]. These engineered Cas proteins had an incredible reduction in off-target activity. Cytosine, instead of adenine, is responsible for an off-target mutation in rice hence needs further improvement in tools like base editors [118]. Moreover, PE is also a reliable tool for reducing off-target mutations e.g., targeting of 179 predicted off-target sites with 12 pegRNAs and nCas9 nickase [91] resulted in 0.00~0.23% of off-target mutations.
- Another limiting factor is the commercial adaptation of genome-edited crops in some countries and has been discussed in detail earlier (see regulatory aspects and risks associated with genome editing). Although the GM crop decision and utilization of GETs is pending, there is great potential for robust, efficient, and environmentally friendly breeding for improved variety development.

- Immune DNA/RNA viruses towards eukaryotes is a crucial limitation of the CRISPR/Cas9 system due to outflow and instant replication of viruses [125]. There is a dire need for a widely acceptable CRISPR version, such as Cas13, to overwhelm this issue. Among all three proteins (Cas13a, Cas13b, and Cas13c), Cas13a is referred for its precise, robust RNA replications, and can exert against RNA viruses [126]. In short, Cas13 would be a better choice for targeting viral RNA against CRISPR/Cas9.
- In case of BE, many obstacles, such as high off-target activity, huge editing window, and limited PAM sites limit its efficiency. Several approaches have been used to minimize these limitations, including application of the REPAIR and RESCUE system in plants, alteration of the CBE and ABE system, generating mutations simultaneously at multiple loci in rice [116], such as multiplex BE for crop improvement. RNP approach had also overcome the regulatory obstacles of base editors by increased efficiency to improve agronomic traits.
- PE also exhibits some key issues, including cell type determinants, state of cell, DNA repair mechanisms deciding the fate of productive or unproductive PE or transport of PE protein or pegRNA for regulation of in vivo applications. This issue could be resolved by manipulating DNA repair favoring to replace the edited strand over the non-edited strand subsequent to successful insertion of a 3′ flap, or alternatively use smaller reverse transcriptase enzymes.

7. Conclusions and Outlook

Significant advancements have been made in genome editing tools, especially the CRISPR-Cas system and its variants. These new GETs have made crop improvement precise, robust, and better than earlier methods (i.e., conventional breeding). Owing to multiple genome editing skills, i.e., insertion, deletions, gene knockout/in, direct substitution at any loci, the CRISPR/Cas system is ruling over the other GETs for the development of ideal plants and crop domestication, i.e., super rice generation. CRISPR/Cas system has been used to develop a variety with desired changes and phenotypes in already existing elite rice varieties, consequently enhanced rice grain quality. Developing versatile approaches, such as CRISPR-based BE, PE, CRISPR transposes, and recombinases would provide exciting opportunities in genome editing. They also highlight a major milestone enabling precise sequence alteration of any desired genome as well as rearrangements of larger DNA sequences. Editing efficiency of PE can be enhanced by using a different transgenic selection system and reverse transcriptase.

The new addition of CRISPR-interference (CRISPRi) and CRISPR-mediated activation (CRISPRa) in the CRISPR family, using the dCas9 toolkit, as developed in maize [127], has great potential for further improvement of genome engineering. Targeting the core promoter of a gene by editing technologies could be a reliable approach for fine-tuning of any desired gene expression, unearthing new avenues for breeding improved rice grain quality. Moreover, novel and potential delivery methods used in the latest approaches for gene editing will produce transgene-free products; hence, overcoming the limitation of ethical, regulatory, and commercialization issues. Utilization of these editing technologies in functional genomics linked with other approaches will allow combating global food challenges and will help to accomplish the zero-hunger goal, one of the sustainable development goals set by the United Nations, by 2030.

On the other hand, it is reported that an autophagy gene *OsATG8b* controls grain quality in addition to candidate genes, including *qGC10* for gel consistency, *qHd2-1* and *qGS-7* for grain size, physical appearance of grain, eating, and cooking quality [128–130]. Similarly, several novel QTL, metaQTL, ortho-MQTL, and candidate genes for grain yield and related traits, drought, salinity, and heat stresses have been identified [131–133]. Employing the latest editing technologies on these candidate genes/QTLs has remarkable potential for fine-tuning rice grain yield, abiotic stress tolerance, grain appearance, and quality improvement in the future breeding program.

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