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CRISPR/Cas 9 tool kit for rice crop improvement: An inclusive review

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Abstract
The consequences of climate change, new fungal infections, the desire for fewer chemicals on the field and high-quality agricultural products are the major challenges for sustainable agriculture. Plant breeders respond to all these challenges with new varieties, using the most suitable breeding methods. In the last decade there has been a dramatic expansion of new genetic engineering and biological tools: genomics data; metabolic engineering; high-throughput analytical tools, including whole organism gene expression analysis and metabolomics and powerful genome-editing technologies. These new breeding methods complement farmer's tool boxes and offer additional opportunities to enhance plant breeding. Among these, the bacterial clustered, regularly interspaced, short palindromic repeat (CRISPR) associated protein 9 (Cas9) or CRISPR-Cas9 has emerged as the easiest, most economic and efficient technology to undertake genome editing. This technique allows precise site-specific gene modification or integration. This review summarizes the application of CRISPR/Cas9 system in rice.

Keywords: Rice, CRISPR, Cas, genome editing

Introduction
The history of plant breeding began with the selection of especially desirable traits. This was followed by hybrid and mutation breeding, later genetic engineering and marker-assisted breeding. These developments were necessary to provide new solutions for the steady increase of requirements from society. These traditional breeding methods have been extremely successful in providing rice varieties with high yields and other improved traits, and even today, they remain the corner stone of plant breeding. Of late, these traditional breeding methods were hastened by increasing selection efficiency through marker-assisted selection (Collard *et al*. 2008) [11] and genomic selection (Desta *et al*. 2014) [14]. Nevertheless, the more knowledge we acquire about the underlying genomic factors of yield and quality, the more the limitations of these traditional breeding methods become apparent. The efficiency of traditional breeding methods is determined by the amount of available functional diversity, which is limited in many elite varieties that have passed through genetic bottlenecks during domestication (Shi *et al*. 2015) [43]. Therefore, the dependence on natural or randomly induced diversity is a limiting factor, which slows down the breeding process (Watson *et al*. 2018) [52] and contributes to an unpredictable breeding outcome (Scheben *et al*. 2018) [40]. On the other hand, the highly precise nature of the genome editing technology CRISPR-Cas9 facilitates an unparalleled level of control over the mutation process, allowing immediate pyramiding of multiple valuable traits into an elite background within one generation (Zhang *et al*. 2014) [59]. Furthermore, direct improvement of selected varieties by genome editing does not introduce potentially deleterious alleles from crossing and recombination. It has arisen as a remarkable strategy for efficient and targeted genome manipulations, particularly for crops which have complex genomes and which are difficult to improve through traditional breeding approaches (Feng *et al*., 2013) [16].

What is CRISPR-Cas9 and how it works?
The CRISPR-Cas9 system is an innovation of plant breeding that utilizes site-directed nucleases to target and modify DNA with great accuracy (Barrangou *et al*. 2007; Horizon Discovery 2016) [5, 20]. Developed in 2012 by scientists from the University of California, Berkeley. CRISPR-Cas9 has received a lot of attention in recent years because of its variety of applications, which include biological research, breeding and development of agricultural crops and animals and human health applications. These comprise gene silencing, DNA-free CRISPR-Cas9 gene editing, homology-directed repair (HDR), and transient gene silencing or

transcriptional repression (CRISPRi) (Barrangou *et al*. 2007; Bolotin *et al*. 2005; Dharmacon 2016; Jinek *et al*. 2012) [5, 6, 15, 23].

CRISPR, or Clustered Regularly Interspaced Short Palindromic Repeats, is an integral part of a bacterial defense system. It is also the basis of the CRISPR-Cas9 system. The CRISPR molecule is made up of short palindromic DNA sequences that are repeated along the molecule and are regularly-spaced. Between these sequences are "spacers", foreign DNA sequences from organisms that have previously attacked the bacteria. The CRISPR molecule also includes CRISPR-associated genes, or Cas genes. These encode proteins that unwind DNA and cut DNA, called helicases and nucleases, respectively (Barrangou *et al*. 2007; Harvard University 2015) [5, 18].

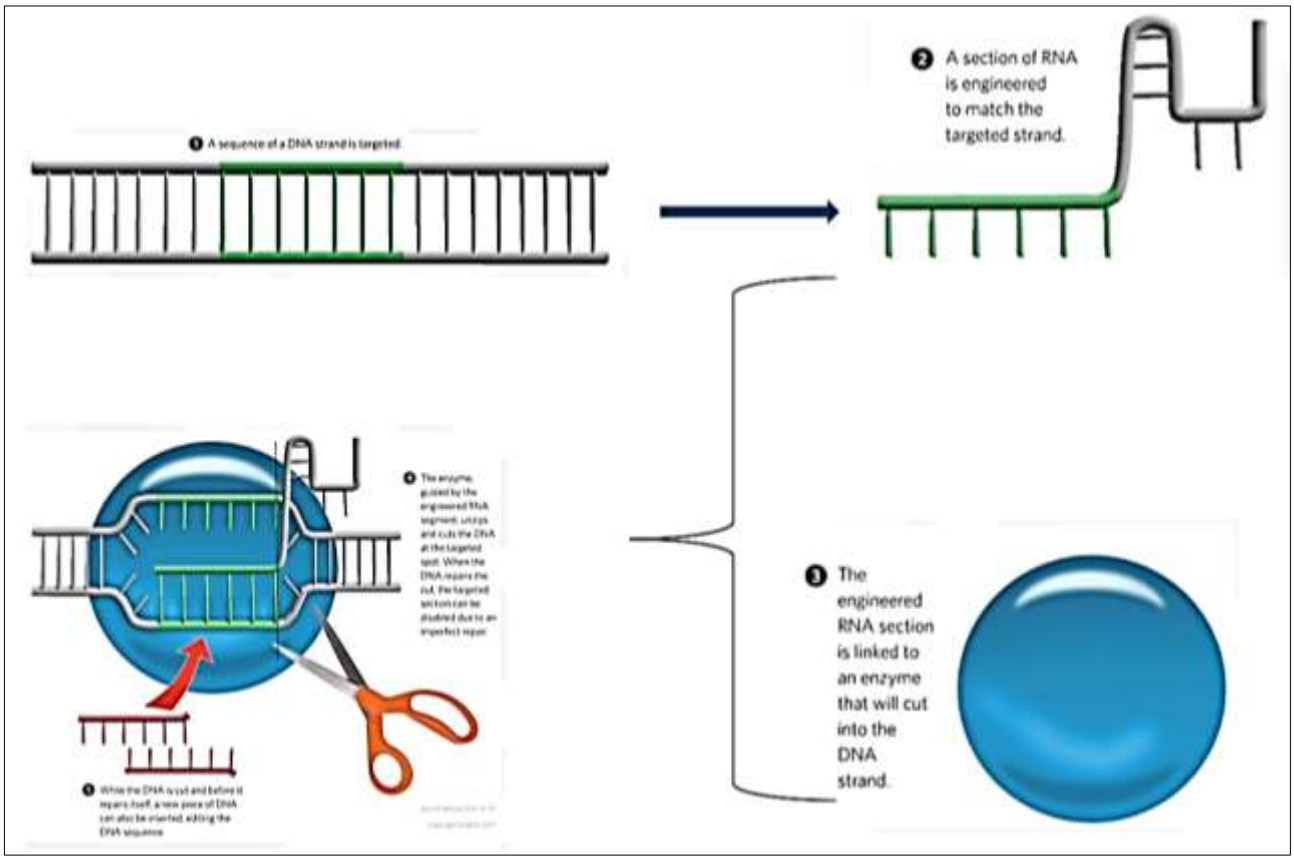

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Fig 1: Five Steps to Gene Editing

The CRISPR immune system protects the bacteria from repeated virus attacks through three steps:

1. **Adaptation:** When DNA from a virus invades the bacteria, the viral DNA is processed into short segments and is made into a new spacer between the repeats. These will serve as genetic memory of previous infections.
2. **Production of CRISPR RNA:** The CRISPR sequence undergoes transcription, including spacers and Cas genes, creating a single-stranded RNA. The resulting single-stranded RNA is called CRISPR RNA, which contains copies of the invading viral DNA sequence in its spacers.
3. **Targeting:** The CRISPR RNAs will identify viral DNA and guide the CRISPR-associated proteins to them. The protein then cleaves and destroys the targeted viral material.

Scientists take advantage of the CRISPR-Cas9 systems' detection of specific DNA sequences and use it in the development of improved crops. In place of viral DNA as spacers, scientists design their own sequences, based on their specific gene of interest. If a gene's sequence is known, it can be easily used in CRISPR. It will then act just like a spacer for the system and guide the Cas9 protein to a DNA matching sequence (Barrangou *et al*. 2007; Harvard University 2015) [5, 18].

CRISPR-Cas9 allows researchers to perform the following

Gene Knock-Out

Gene silencing using CRISPR begins with the use of a single guide RNA (sgRNA) to target genes and initiate a double stranded break by means of the Cas9 endonuclease. Then an innate DNA repair mechanisms, the non-homologous end-joining (NHEJ) repairs these breaks. However, NHEJ is error-prone and results in genomic deletions or insertions, which then translates into permanent silencing of the target gene (Dharmacon 2016; Cong *et al*. 2013; Add Gene 2014) [15, 12].

DNA-Free Gene Editing

CRISPR can be used for DNA-free gene editing without the use of DNA vectors, requiring only RNA or protein components. A DNA-free gene editing system can be a good choice to avoid the possibility of undesirable genetic alterations due to the plasmid DNA integrating at the cut site or random vector integrations (Dharmacon 2016; Cong *et al*. 2013) [15, 12].

Gene Insertions or "Knock-ins"

The CRISPR-induced double-strand break can also be used to create a gene "knock-ins" by using the cells' homology-directed repair. The precise insertion of a donor template can alter the coding region of a gene. Earlier studies have revealed that single-stranded DNA can be used to create precise insertions using CRISPR-Cas9 system (Dharmacon 2016; Cong *et al*. 2013; Add Gene 2014) [15, 12].

Transient Gene Silencing

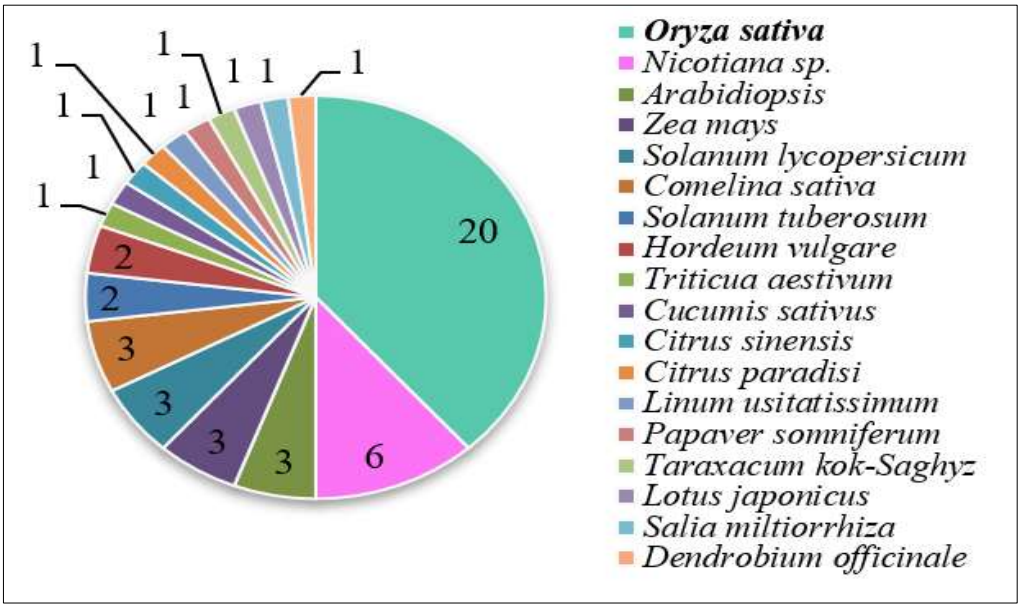
By altering the Cas9 protein so it cannot cut DNA, transient gene silencing or transcriptional repression can also be done. The modified Cas9, led by a guide RNA, targets the promoter region of a gene and reduces transcriptional activity and gene expression. Transient activation or up regulation of specific genes can be efficiently done (Dharmacon 2016; Cong *et al*. 2013) [15, 12].

Why we consider genome editing?

By the year 2050, more or less ten billion people will inhabit the earth. This is almost twice as many as in 1990. The world's growing population presents agriculture with enormous challenges. Sustainable land use is now more important than ever. As are steady and stable yields. This objective necessitates progress in the area of plant breeding. Crops such as rice, wheat and maize have been sustaining people for many centuries. But changing environmental conditions and diverse lifestyles have always challenged humanity to use the innovations of their time to adapt agriculture to new nutritional requirements.

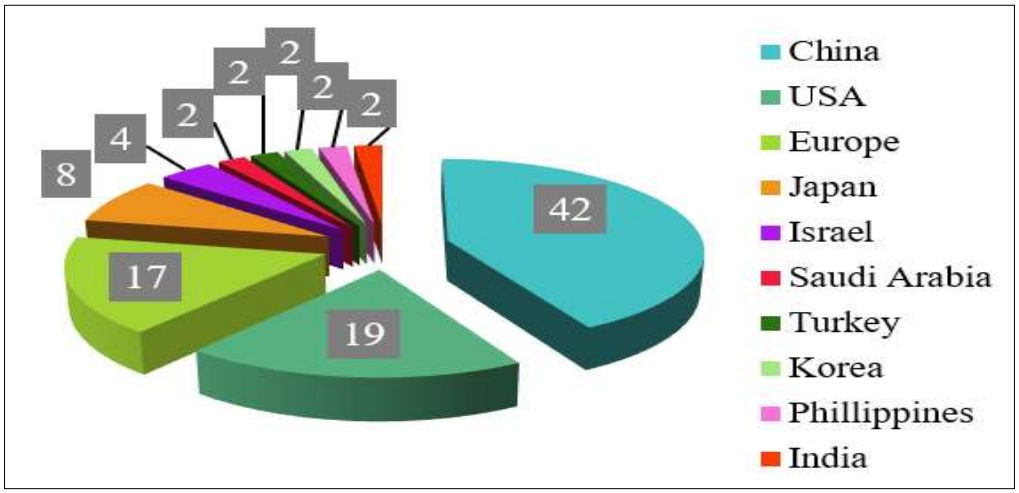
How can genome editing can help us?

The stability and simplicity of use presented by new breeding methods offers unique possibilities. They enable the development of new, high-yielding varieties adapted to their environment. Increasing problems caused by climate change, biotic and abiotic stresses and degradation of arable land can be more effectively mitigated, while simultaneously, ensuring food production in adequate quantities at affordable prices.



Source: Ricroch A, Clairand P, & Harwood W. 2017. Use of CRISPR systems in plant genome editing: Toward new opportunities in agriculture. Emerging Topics in Life Sciences, 1(2): 169-182.

Fig 2: Plant species studied in articles with agricultural applications (2014-2017).



Source: Ricroch A, Clairand P, & Harwood W. 2017. Use of CRISPR systems in plant genome editing: Toward new opportunities in agriculture. Emerging Topics in Life Sciences, 1(2): 169-182.

Fig 3: CRISPR studies by country

CRISPR-Cas9 in rice crop improvement

Initially the applications of genome editing was reported in the model species *A. thaliana* and *N. benthamiana* (Feng *et al*. 2013; Li *et al*. 2013; Nekrasov *et al*. 2013) [16, 26, 37], along with crops such as rice (Jiang *et al*. 2013; Miao *et al*. 2013; Shan *et al*. 2013; Wang *et al*. 2016) [35, 22, 41, 51], wheat (Shan *et al*. 2013; Wang *et al*. 2014) [41], maize (Char *et al*. 2016), potato (Butler *et al*. 2015) [7], and tomato (Pan *et al*. 2016) [38]. In the last few years, CRISPR/Cas9 has been used to produce new rice varieties with improved traits, which include improved disease resistance and other stress tolerance, nutritional quality and increased yield.

Rice Mutant Libraries

The completion of whole genome sequence of rice in 2004, presented a great task of analyzing and assigning functions to all the predicted genes in the genome which could be priority in rice functional genomics studies (Wang *et al*. 2013) [49]. The introduction of CRISPR/Cas9 system makes the development of such mutant libraries easier. In rice a high quality large-scale CRISPR/Cas9 mutant library has been constructed to study gene functions (Meng *et al*. 2017) [34]. To construct the mutant library 12,802 genes highly expressed in rice shoot base tissue and 25,604 corresponding sgRNAs were selected. In future, many such rice mutant libraries are likely to be constructed, that would help to identify gene function and accelerate genetic improvement.

Yield and Quality Improvement

Yield and quality are quantitative characters and under the control of multiple genomic loci. Numbers of panicles per plant, number of grains per panicle and grain weight are three major component traits that determines yield in rice (Xing and Zhang 2010) [53]. CRISPR has been used to edit the genome of rice. The team of Ying Wang from Syngenta Biotechnology China designed several CRISPR sgRNAs and successfully deleted fragments of the dense and erect panicle1 (DEP1) gene in the Indica rice line IR58025B. Improvements in yield-related traits, such as dense and erect panicles and reduced plant height, were observed in the mutant plants produced. The desired rice cultivars with improved grain number, dense erect panicles and larger grain size were developed by editing the four genes Gn1a, DEP1, GS3, and IPA1separately with the help of the CRISPR/Cas9 system (Li *et al*. 2016) [27-28]. Using CRISPR/Cas9 three genes associated with grain weight i.e., 2 (GW2), GW5 and thousand grain weight 6 (TGW6) were edited simultaneously and 29.3% increase in 1000 grain weight was recorded in triple null mutant (Xu *et al*. 2016) [55-56]. This put forward that pyramiding of major yield associated genes in a single cultivar through multiplex gene editing would be significant in improving the yield components of rice. In a study, genes GS3, DEP1, GS5, GW2, Gn1a, and TGW6, identified to be negative regulators of grain size and number and grain weight were knocked out to improve rice yield (Zhang *et al*. 2017) [61-62]. Another agronomic trait with significant effect on yield in rice is heading date. Three major genes (Hd2, Hd4 and Hd5) having negative affect on heading date in rice were targeted and mutagenized with CRISPR/Cas 9-mediated multiplex genome editing tool. The heading date was significantly shortened in nine mutated rice lines (Li *et al*. 2017). Several pioneer rice hybrids have shown a yield advantage of around 20% over inbred lines. Eleven novel TGMS indica rice lines were developed through CRISPR/Cas9 system within only 1 year (Zhou *et al*. 2016) [63]. Further, in japonica rice a prominent locus Carbon Starved Anther (CSA) gene, that show male sterility under short day conditions and male fertility under long day conditions was targeted and edited with CRISPR/Cas9 technology to develop two reverse PGMS lines 9522csa and JY5Bcsa and one rP(T)GMS145 line KY131csa-4 (Li *et al*. 2016) [27-28]. This suggested that CRISPR/Cas9 can accelerate two line hybrid rice breeding. CRISPR/Cas9 system was used to knock out three genes GW2, GW5 and TGW6 simultaneously and resultant triple mutant rice showed significant increase in thousand grain weight (Xu *et al*. 2016) [55-56]. During storage, grain deterioration is a major cause of reduction in quality and seed longevity leading to economic losses. In the indica rice line, Zhonghua 11 the fragrant gene Badh2 has been edited using CRISPR/CAS9. The mutated line had an additional T base in the first exon of Badh2 and resulted in increased amount of 2AP and improved fragrance in rice (Shao *et al*. 2017) [45]. In rice growth and yield are regulated by a number of phytohormones and their corresponding signaling networks. Abscisic acid (ABA), an important phytohormone, is perceived by the soluble pyrabactin resistance 1 (PYR1)/PYR1-like (PYL)/regulatory components of the ABA receptor (RCAR) family proteins. CRISPR/Cas9 technology has been efficiently utilized to edit group I (PYL1-PYL6 and PYL12) and group II (PYL7-PYL11 and PYL13) PYL genes in rice leading to increased growth and productivity in rice. Rice growth was promoted by the mutation of group I genes (PYL1-PYL6 and PYL12) and among them pyl1/4/6 showed the most vigorous growth and improved grain productivity, while maintaining near-normal seed dormancy and other agronomic traits (Miao *et al*. 2018) [36]. Several important traits such a yield and abiotic stress tolerance are controlled by two or more genes. In crop improvement programs, many studies have make an effort to map these quantitative regions (quantitative trait loci-QTL) regulating agronomically important traits. The identified QTL regions were introgressed into elite lines to develop improved varieties. But, this introgression is tedious if the QTLs are linked closely and introducing non-target regions into elite line may cause deleterious effects. CRISPR/Cas9 system can be a potent tool to establish and study rare mutations in crop plants. In rice varieties the function of grain size (GS3) and grain number QTLs (Gn1a) were studied using a CRISPR based-QTL editing approach (Shen *et al*., 2018) [42]. The study revealed that the same QTL can have highly varied and contrasting effects in different backgrounds. A new study reported the production of clonal diploid seed in rice by combining ectopic expression of *BABY BOOM1* with a CRISPR/Cas9 mediated triple mutant that converts meiosis to mitosis (Matthew R. Willmann 2019) [33].

Nutritional Improvement

Rice is the principal food crop accounting for the supply of 70% of daily calories for more than half of the world population. The risk of life-threatening diseases such as hypertension, diabetes, and colon cancers can be reduced by the intake of rice with high amylase content (AC) and resistant starch (RS). Therefore, rice varieties with high AC and RS need to be developed to improve human health (Chen *et al*. 2012) [9]. CRISPR/Cas9 tool has been effectively used to develop high amylose rice by targeting two rice branching enzyme (SBE) SBE I and SBE IIb (Sun *et al*. 2017) [46].

Though the sbe I mutants and wild types did not show any significant variations, sbe IIb mutants exhibited significant increase in AC and RS content. This suggested that SBE IIb is crucial in determining the fine structure and nutritional properties of starch and editing of SBE IIb with CRISPR/Cas9 would be significant in the development of rice varieties with high amylase and RS content. Cadmium (Cd) is a toxic heavy metal which causes serious health effects in people consuming rice. Indica cultivars have more Cd content than japonica rice cultivars and need to be reduced for food safety (Grant *et al*. 2008) [17]. Several measures like soil treatment, phytoremediation, field flooding and charcoal application have been assumed to decrease the Cd level but they were effective only to some extent. The development of low Cd rice cultivars through conventional breeding is highly challenging and new approaches for developing Cd free rice lines are essential for public health. CRISPR/Cas9 can be used to develop non-toxic and healthier rice varieties by modifying genes. Recently CRISPR/Cas9 system has been used to create indica rice lines with low Cd by knocking out the metal transporter gene Os Nramp5 (Tang *et al*. 2017) [48]. The mutated indica rice lines were tested in field trials and results revealed that the OsNramp5 grains had less than 0.05 mg/kg Cd concentration as compared to the wild-type indica rice with 0.33 to 2.90 mg/kg Cd concentrations in grains without any effect on the plant yield. It is expected that the genome editing systems would be used in near future to reduce the risks of multiple heavy metal contamination in rice grains. To increase β-carotene content in rice endosperm, five rice carotenoid catabolic genes (OsCYP97A4, OsDSM2, OsCCD4a, OsCCD4b, and OsCCD7) were knocked out using CRISPR/Cas9system (Yang *et al*. 2017) [57]. But these targeted mutations failed to increase carotenoid accumulation in rice seeds and needs further investigations.

Biotic Stress Tolerance

The major causes of reduction in yield and quality of rice are biotic agents such as bacteria, fungi, viruses and insects (Heinrichs and Muniappan 2017) [19]. Advances in genome editing tools have opened new ways to enhance resistance in crops. In recent years, the CRISPR/Cas9 system has been employed to respond to several agricultural challenges, including the achievement of improved biotic stress resistance (Arora and Narula 2017) [2]. The genome editing methods has been used to edit genes associated with diseases to improve disease resistance in rice. In rice a vascular disease, Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. Oryzae (Xoo) is a major threat for global food security. Considerable efforts have been made to develop BLB resistant rice varieties through conventional and molecular breeding methods (Das *et al*. 2018) [13]. However, the rapid emergence of new virulent pathotypes has led to the development of advanced methods for reducing blight disease. Transcription activator-like effectors (TALEs), the type III effector proteins from *Xanthomonas* species, usually target the SWEET gene family, the sugar transporters that release the sugar into the apoplast of rice cells (Cohn *et al*. 2014) [10]. TALEN technology was applied to disrupt the bacterial protein binding sequence in the promoter of OsSWEET14 for conferring resistance against bacterial blight (Li *et al*. 2012). Likewise, CRISPR/Cas9 technology has been used to create a null mutation in OsSWEET13 to stop its neutralization by the TAL effector gene pth Xo2 resulting in improved resistance against bacterial blight disease in indica rice, IR24 (Zhou *et al*. 2015) [64]. Recently, in the Os09g29100 gene promoter the EBEtal7 binding site was modified with TALEN technology to reduce Tal7 binding, which could potentially decrease BLB disease severity in rice (Cai *et al*. 2017) [8].

Previous studies stated that the knockdown of Os8N3 resistance to *Xanthomonas oryzae* pv. oryzae (Xoo) in rice plants, resulted in enhanced resistance while displaying abnormal pollen development. The CRISPR/Cas9 system has been used to knockout rice Os8N3, so as to confer enhanced resistance to Xoo. The transmission of mutations was confirmed from the genotypes and edited Os8N3 analysis in T_0, T_1, T_2, and T_3 transgenic rice plants. Segregation in the T_1 generation showed the stable transmission of CRISPR/Cas9-mediated Os8N3 gene editing without the transferred DNA (T-DNA) (Young-Ah Kim *et al*. 2019) [58]. Therefore, the CRISPR/Cas9-mediated Os8N3 edition can be effectively utilized for non-transgenic crop improvements.

The most destructive disease of rice is rice blast caused by ascomycetes fungus *Magnaporthe oryzae*. Under favorable environmental 60-100% yield losses due to blast incidence were estimated (Kihoro *et al*. 2013) [24]. Though, several efforts have been made to develop blast resistant cultivars using advanced molecular and genomic tools, conventional host resistance breeding has played a significant role in developing new blast resistant cultivars (Ashkani *et al*. 2015) [3]. But, conventional breeding is highly time consuming and laborious. Moreover, the emergence of new pathogenic variability often causes a breakdown of resistance cultivars. Therefore, recently developed engineered nucleases could be a suitable alternate for enhancing the resistance of rice to *M. oryzae*.

Rice plants resistant to rice blast disease were developed through CRISPR/Cas9 targeted knockout of OsERF922 and OsSEC3A genes in rice (Wang *et al*. 2016; Ma *et al*. 2018) [51]. The mutant plants with disrupted OsSEC3A gene in a putative subunit of a complex engaged in exocytosis, unveiled a pleiotropic phenotype comprising enhanced resistance against *Magnaporthe oryzae*, higher levels of salicylic acid (SA) content, up-regulation of pathogenesis- and SA related genes and dwarf stature (Ma *et al*. 2018). On the other hand, this change in agronomic traits was not observed inT_1 and T_2 transgene free plants mutated in the ethylene responsive factor (ERF) 922, a transcription factor implicated in multiple stress responses. Reduced number of blast lesions was observed in mutant plants at both seedling and tillering stages (Wang *et al*. 2016) [51]. On the whole, these findings demonstrated the power of CRISPR/Cas9 system against fungal diseases and its usefulness in developing disease resistant cultivars. Another important rice disease with a severe effect on rice production is rice tungro disease (RTD). The interaction of two different viruses' rice tungro spherical virus (RTSV) and rice tungro bacilliform virus (RTBV) is responsible for this disease. Extensive breeding for resistance development and assessment of resistant near isogenic lines revealed that the translation initiation factor 4 gamma (eIF4G) gene is involved in RTSV resistance and the YVV residues of eIF4G are found to be associated with the reactions to RTSV (Lee *et al*. 2010) [25]. Of late, new sources of resistance to rice tungro spherical virus (RTSV) were developed through CRISPR/Cas9 mediated mutagenesis of eIF4G alleles in rice plants (Macovei *et al*. 2018) [32]. In the RTSV-resistant T_2 plants mutations were not detected in the off-target sites and were

negative when tested for the presence of Cas9. Additionally, the RTSV inoculated plants showed enhanced agronomic parameters such as plant height and grain yield when, compared to their wild-type counter parts under glasshouse conditions. Further, CRISPR/Cas9 tool can be used in stacking process. Unlike the introgression of conventional resistance genes, the system avoids genetic drag on neighboring regions with potentially negative effects on agronomic performance. It also allows the simultaneous establishment of multiple resistances in a single generation by multiplexing, i.e., the parallel use of several sgRNAs targeting different genes.

Abiotic Stress Tolerance

In rice bentazon sensitive lethal (BEL) gene is responsible for resistance to bentazon and sulfonyl urea herbicides (Pan *et al*. 2006) [39]. In two-line hybrid rice production, bel mutants are often used to select seed contaminants. Though BEL is important in determining hybrid rice production safety, its use is always restricted due to inadequate natural genetic resources. So, a CRISPR/Cas9- mediated mutation of BEL gene was examined in rice using the Agrobacterium-mediated gene transfer (Xu *et al*. 2014) [54]. Stable CRISPR/Cas9 transformants showed 2-16% mutagenic efficiency whereas the phenotypic analysis unveiled that the biallelic mutated transgenic plant was sensitive to bentazon.

Of late, herbicide tolerant rice varieties were developed through genome editing-based mutations of ALS gene (Li *et al*. 2016; Sun *et al*. 2016) [27-28, 47]. Multiple discrete point mutations were introduced in the rice ALS gene using CRISPR/Cas9. Phenotypic screening showed that the wild-type plant died after 36 days of bispyribac sodium (BS) spraying, while the mutated lines showed tolerance to BS and grew normally (Sun *et al*. 2016) [47]. Therefore, using genome editing homozygous herbicide tolerance rice plants can be developed within one generation. Previous studies reported that TIFY1b, a transcription factor, is one of the genes involved in cold tolerance in rice. CRISPR/Cas9 technology has been used to edit the TIFY1b and its homology gene TIFY1a (Huang *et al*. 2017) [21]. Site-specific mutations were detected in T_0 rice plants. The results indicated the TIFY1 mutant lines could be used to study the role of TIFY1 genes in rice adaptation to low temperature. Activation-induced cytidine de aminase (Target-AID) method was used to carry out base editing of the herbicidal gene, C287 in rice. In this dCas9 combined with cytidine deaminase was used for base editing without introduction of DSBs (Shimatani *et al*. 2017) [44]. BE3 base editor is an advanced genome editing tool that joins nicked cas9 (ncas9- a D10 mutation in cas9), cytosine deaminase and the uracil glycosylase inhibitor (UGI) which inhibits base-excision repair. In rice OsPDS and OsSBEIIb genes were edited with BE3 base editor (Li *et al*. 2017a). This study showed the successful application of base editing in rice. Anning Zhang *et al*., 2019 attempted to improve the rice salinity tolerance by engineering a Cas9-OsRR22-gRNA expressing vector, targeting the OsRR22 gene in rice. At the seedling stage, the T_2 homozygous mutant lines showed an enhanced salinity tolerance compared to wild-type plants. This study indicated that CRISPR/Cas9 is a valuable approach to enhance the salinity tolerance of rice.

CRISPR/Cas9 has been the fastest growing genome editing tool up to now. It has been proved to be efficient and precise in genome editing. Nevertheless Cas9 has certain limitations. CRISPR/Cpf1 (Cpf1) has been found as an alternative approach that can overwhelmed some of those limitations. Cpf1 allows targeting in AT-rich region, creating a staggered cleavage, and cutting at the distal end to the PAM (Protospacer Adjacent Motif) regions. At IRRI the efficiency of Cpf1 system was successfully tested in rice using OsEPFL9 which is a developmental gene known to regulate the stomatal density in leaf. The Exon1 of OsEPFL9 was targeted and the knockout lines were studied for several generations for establishment of stabilized editing, as well as transmission and segregation of edits through generations. The usage of Cpf1 as a genome editing tool to manipulate stomatal patterning may further help to get more understanding of the physiology of rice in stress conditions (IRRI, 2019).

Conclusion

The CRISPR/Cas9 system is modern and exciting add-on to the genome editing toolbox owing to its simplicity and successful application. The wide array of CRISPR/Cas9 optimizations allows the system to be used for multiple purposes with increasing efficiency and specificity. Latest technologies facilitating epigenetic and transcriptional regulation should complement more traditional methods and could be used to tune or multiplex plant gene expression with exceptional control. The ability to base edit in plants is in its infancy but, considering the potential applications for targeted *in vivo* substitution mutagenesis, represents an exciting opportunity. CRISPR/Cas9 and associated genome editing tools have brought a revolutionary change in rice improvement which is critical for meeting the demands and ensuring the prerequisite of rice for future generations. Given the broad interest and push in CRISPR systems, new approaches to increase efficiency, specificity, heritability and new CRISPR variants and applications are unavoidable in the near future. It is an exciting time to be involved in plant research.

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