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CRISPR mediated genome engineering to develop climate smart rice: Challenges and opportunities

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ABSTRACT

Rice is a staple food crop, which ensures the calorie requirement of half of the world's population. With the continued increase in population, rice will play a key role in achieving the food security. However, in the constantly shrinking scenario of rice fields, the necessity of these extra grains of rice must be met by reducing the yield loss due to various abiotic and biotic stresses. The adverse effects of climate impact both quality and quantity of rice production. One of the most desirable applications of CRISPR/Cas technology would be to develop climate smart rice crop to sustain and enhance its productivity in the changing environment. In this review, we analyze the desirable phenotypes and responsible genetic factors, which can be utilized to develop tolerance against major abiotic stresses imposed by climate change through genome engineering. The possibility of utilizing the information from wild resources to engineer the corresponding alleles of cultivated rice has been presented. We have also shed light on available resources for generating genome edited rice lines. The CRISPR/Cas mediated genome editing strategies for engineering of novel genes were proposed to create a plant phenotype, which can face the adversities of climate change. Further, challenges of off-targets and undesirable phenotype were discussed.

1. Introduction

Rice is the primary staple food crop for more than half of the world's population and remains the largest calorie provider. Rapid growth of human population continues to demand higher production of food grains. Various abiotic stresses such as drought, flooding, salinity, heat, cold, ion toxicity and radiation reduce both yield and quality of agricultural production. The predicted climate change is likely to result in extreme drought and temperatures posing a serious threat to global food production. Temperature and precipitation are major environmental factors, which influence rice growth, development, and productivity [1]. At least 23 million ha of rice area is estimated to be drought-prone only in Asia [2]. The global average surface temperature rose by 0.85 °C in the last century posing an alarm for rice cultivation in various parts of the world [3]. Without CO_2 fertilization, effective adaptation, and genetic improvement, the global rice production has been predicted to decrease by 3.2% per degree-centigrade rise in global mean temperature [4]. As the mean temperature of earth surface continues to rise, the precipitation will decrease in the subtropics and extreme events, such as drought, flood, and cyclones are likely to become more frequent [3]. The adverse effects of climate will not only compromise the rice yield and productivity but also will have larger impact on society due to increased prices, hunger, unemployment, and poverty, specifically in Asia and Africa. Therefore, a comprehensive strategy for mitigating the potential impact of climate change in rice cultivation is imperative through designing the climate-smart genotypes.

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-mediated genome editing is a versatile tool that has opened a new avenue to precisely modify both animal and plant genomes. The CRISPR technology, which started just about 5 years ago, has revolutionized the ability of researchers to study the gene function and positively alter it to improve abiotic and biotic stress tolerance, increase yield potential of crop plants or even to improve the quality of grains. The CRISPR system, initially discovered in primitive bacteria, provides acquired immunity against bacteriophages. Short segments of foreign DNA, termed as 'spacers', get integrated into the bacterial genome, which consequently get transcribed and processed into CRISPR RNA (crRNA). The crRNA anneals to the trans-activating crRNAs (tracrRNAs) to form the guide RNA (gRNA) that directs the Cas9 (CRISPR associated protein 9) endonuclease to the target site. The target recognition strictly

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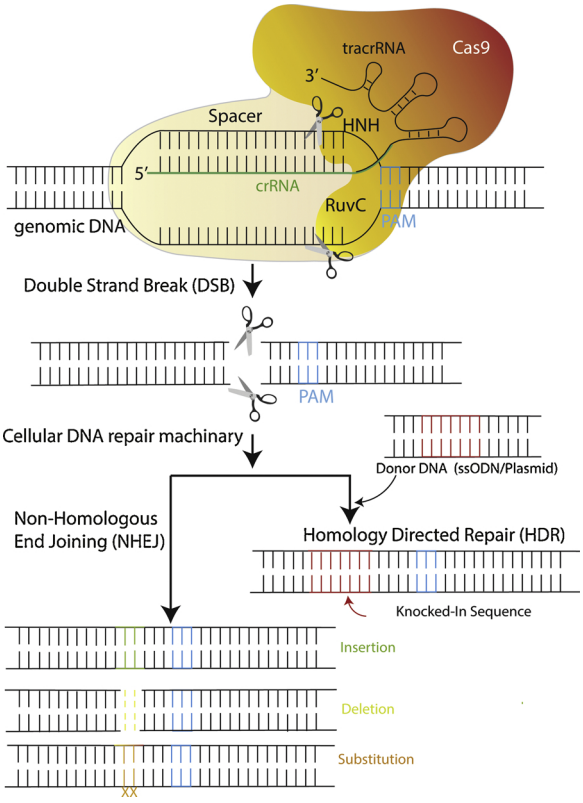

Fig. 1. CRISPR/Cas9 mediated genome engineering.
The T-DNA carrying the expression cassettes for the Cas9 endonuclease and the sgRNA gets randomly integrated in the host genome. Upon the gene expression, the sgRNA directs the Cas9 enzyme to the target site and guides Cas9 to introduce double strand break (DSB). The HNH-like nuclease domain of Cas9 cleaves the DNA strand complementary to the gRNA sequence (target strand), while the RuvC-like nuclease domain cleaves the nontarget strand. Mutation in one of these two domains will convert the Cas9 enzyme to a nickase while mutating both domains will create the dCas9 enzyme without nuclease activity. Both nickase and dCas9 have several important applications in functional genomics. The DSB can be repaired by the host cell's repair machinery by the error-prone Non-Homologous End Joining (NHEJ) method leading to mutations. On the other hand, a new DNA segment (donor) flanked by two homology arms can be introduced at the DSB site by Homology Directed Repair (HDR) method. This provides the basis for precise genome editing to rectify mutations or to introduce a completely new copy of the gene, promoter or other regulatory elements. ssODN: Single stranded oligo deoxynucleotides.

requires the presence of a consensus dinucleotide-containing (NGG or NAG) protospacer adjacent motif (PAM) immediately following the spacer sequence. The RNA-guided endonuclease (RGEN) Cas9 makes a sequence-specific double strand break (DSB) 3-bp upstream of the PAM. This activates host's natural cellular repair machinery to re-ligate the ends generally through Non-Homologous End-Joining (NHEJ) (Fig. 1). The NHEJ is error prone and usually introduces small insertions–deletions (INDELs) or substitution of one or few bases at the cleavage site. This alteration can lead to frameshift mutations that can result in introduction of stop codons and premature release of polypeptide. The crRNA and tracrRNA can be combined and expressed as a single guide RNA (sgRNA) [5]. Thus, the Cas9 can be easily targeted to any genomic sequence in plants by altering the 20 bp spacer sequence of the gRNA [6]. Proliferating cells can also repair the DSB by homology-directed repair (HDR). However, the HDR requires a separate donor template DNA with homologous flanking sequences (homology arms) that match the corresponding ends around the site of the DNA break. The HDR can facilitate repair or replacement of any mutated gene or inferior allele with an appropriate copy of the same gene to improve a trait or to confer a new trait. Thus, the Cas9 endonuclease, coupled with an artificial guide RNA, can be targeted at any DNA stretch of 20 nucleotides followed by its PAM (5′ - $N_{1\text{-}20}$ NGG/NAG – 3′). In this review, we have discussed the potential candidate genes and pathways that can be exploited by the CRISPR/Cas technology to develop climate-smart rice, available resources and recent progress made in generating genome edited rice lines. We have also discussed about the challenges and future prospects of rice genome engineering.

2. Resources available for CRISPR-mediated rice genome engineering

Though CRISPR technology started within this decade only, it has been broadly embraced in all sections of biology including bacteria, animals and plants due to its ease of application, speed, efficiency and heritable nature. Original system involved transforming two vectors carrying the expression cassettes for Cas9 and SgRNA separately [7]. Recently many single binary vectors have been developed carrying both [8]. Many of these binary vectors can be found and ordered from AddGene, a non-profit plasmid repository https://www.addgene.org/crispr/plant/. The only limitation of this technology is to find a unique 20-nucleotide spacer sequence followed by the PAM. More than two dozens of bioinformatic tools are available to design the guide RNA in different organisms [9]. However, only few of these support designing sgRNAs for rice. The CRISPR-PLANT server https://www.genome.arizona.edu/crispr/CRISPRsearch.html uses the MSU Id of rice genes or the chromosomal location of the target region to predict highly specific spacer sequences [10]. The exonic/intronic nature of the spacer, restriction site at the DSB locus (3-nt upstream of PAM) can also be observed. The CRISPR-P 2.0 < http://crispr.hzau.edu.cn/CRISPR2 > is another freely available versatile tool to design plant sgRNA with minimal off-target potentials [11]. In addition to the most widely used *Streptococcus pyogenes* Cas9 (spCas9), this server supports the design of guide sequences for Cpf1 [12,13] and various other Cas9 endonucleases [14–16]. It also provides a comprehensive analysis of the guide sequence including the GC content, restriction endonuclease site, microhomology score and the secondary structure. The spacers can be commercially synthesized as oligonucleotides and inserted into the binary vectors by conventional cloning or more commonly through Golden Gate cloning system for genetic transformation. If the spacer sequence was carefully selected to have a restriction site at the target cleavage site, mutant targets can be PCR amplified and checked by restriction digestion. Transgenic lines can also be screened for targeted mutation using endonucleases such as T7 endonuclease I (T7EI) and Surveyor nuclease that cleave heteroduplex DNA at mismatches and extra helical loops formed by single or multiple nucleotides [17]. For heterozygous lines, Sanger sequencing of the target region shows overlapping peaks due to presence of INDELs. Web applications like CRISP-ID [18], Poly Peak Parser [19], TIDE [20] and ICE < https://ice.synthego.com > can be used for sequence trace decomposition and to get a quantitative estimation of mutation level in the plant. Placement of Locked Nucleic Acid (LNA™) (Exiqon, USA) monomers in oligonucleotides can ensure Tm discrimination between closely related sequences down to as little as one nucleotide difference. LNA-based qPCR and dPCR assays can also be used for rapidly quantifying the extent of on-target genome editing and detecting recombinant mutations [21].

3. Opportunities for genome engineering of rice towards conferring abiotic stress tolerance

Abiotic stresses are complex and so are the tolerance mechanisms. Generally, tolerance to abiotic stress is governed by multicomponent gene networks involving signaling, regulatory and metabolic pathways to restore cellular homeostasis [22,23]. Pyramiding multiple genes for incorporating abiotic stress tolerance trait in rice is expensive, labor intensive and lengthy process. The multiplex genome editing by CRISPR/Cas9 system offers an easy and efficient method that has been

implemented in several plant species including rice [24,25]. The CRISPR mediated repair or replacement of faulty genes using HDR method can be compared to gene introgression without linkage drag. CRISPR/Cas system can be implemented to modify various genes and pathways regulating abiotic stress tolerance to develop climate smart rice, as discussed below.

3.1. Desirable phenotypes of rice suited for climate change

Rice is grown in most diverse environments, i.e., low land, upland, submerged, rain-fed, irrigated, hills, tropical, and temperate regions. Each of these ecologies pose different challenges to rice cultivation. Hence, designing of genotypes should be specific and need-based. In general, cold, heat, and drought stress tolerance at seedling and reproductive stages are desirable. Secondary traits like root and flag leaf characteristics can help in developing cultivars with improved drought and heat tolerance [26]. Enhancing the culm strength to make rice more tolerant to lodging would be an essential feature for regions with threat of submergence and storms [27]. Early maturing short duration traits are useful if crop fails at early stage due to extreme weather. Briefly, the desirable phenotype would largely depend on target rice cultivar, ecology, consumers' and farmers' preferences, and the probable climate challenges.

3.2. Candidate genes and pathways to develop climate smart rice

In rice, several genes and QTLs imparting drought tolerance have been identified and characterized [28]. Some of the positive regulator genes of drought tolerance include an ABA receptor (OsPYL), two cuticular wax accumulation genes (DWA1 and ROC4) and few transcription factors (TF) (OsNAC2, OsNAC14, OsLG3, OsDRAP1) while Drought Hypersensitive (DHS), OsbZIP46 TF, and MODD act as negative regulators [29–35]. Variations in OsLG3 TF promoter created gain of function allele indicating that promoters can also be target of genome editing.

Though rice is a semi-aquatic plant that can survive in standing water, the submergence of complete plant for long time affects the rice growth and productivity immensely. The Sub1A is most widely deployed gene for submergence tolerance trait in rice. The tolerant allele of Sub1A gene has a SNP, which results in P186S substitution in the susceptible allele [36]. SNORKEL1, SNORKEL2, SD1 (SEMIDWARF1), and OsEIL1a are important genes contributing to flood tolerance through SUB1A independent mechanism [37]. Leaf Gas Film 1 (LGF1) facilitates photosynthesis under submergence by the development of gas films on hydrophobic leaves [38]. Sub1A provides an excellent opportunity to utilize the base editing (discussed in Section 4.1.2) for creation of alleles with higher tolerance while other genes are ideal candidates for either introducing better alleles or modification through HDR/NHEJ based gene editing.

Besides submergence and drought, extremes of temperature are other devastating effects of climate change on rice cultivation in tropical and subtropical regions. Transcription factors OsDREB1A, regulator of G-protein signaling COLD1, F-box protein Ctb1, leucine rich repeat receptor like kinase CTB4a and a hypothetical protein qLTG 3-1(Os03g0103300) have been identified to positively regulate cold tolerance [39–42]. Variation in promoter sequence of a hypothetical gene Os09g0410300 has been shown to discriminate between cold tolerant and susceptible varieties [43]. Though, several QTLs for heat stress tolerance have been identified [44–46], the research towards dissection of QTLs to genes and their further structural and functional characterization has not been up to the mark. Homolog of Arabidopsis receptor like kinase ERECTA (ER) (Os06g0203800) and a Thermo-tolerance 1(TT1) (Os03g0387100) are important genes contributing to heat stress tolerance in rice during seedling and reproductive stages [47,48]. Similarly, OsMADS87 was suggested to be negative regulator of seed size during heat stress [49]. Considering the adverse effects of high temperature in rice productivity, more genes associated with heat stress tolerance need to be functionally characterized by utilization of gene editing. Besides the protein coding genes, microRNAs (miRNAs) can also be used as targets of editing to improve the abiotic stress tolerance [50,51]. An additional list of genes important for rice abiotic stress tolerance have been enumerated in table S1.

3.3. Wild species of rice: repository of useful genes associated with hardy characters

Wild rice species are valuable and rich depository for tolerance traits. Many abiotic stress tolerance genes have been identified in wild species. The *O. officinalis, O. nivara,* and *O. glaberrima* are good genetic resources for abiotic stress tolerance [52,53]. Transcription factors identified in *O. rufipogon* genome increased the salt tolerance of rice by positively regulating K^+ homeostasis [54]. The *O. rhizomatis* and *O. eichingeri* accessions showed submergence tolerance independent of SUB1A, which is indicative of a different mechanism in place [55]. Similarly, CBF3/DREB1G and COLD1 genes derived from *O. rufipogon* showed tolerance to low temperature [56]. An early-morning flowering (EMF) locus has been identified from *O. officinalis,* which is an important trait to mitigate heat-induced spikelet sterility [57]. Given the fact that wild rice species are best adapted to environmental stresses and extreme weathers, they can provide novel and significant clues to develop climate smart rice cultivars. CRISPR mediated genome editing will not only help to understand the genetic basis of hardy characters of wild rice varieties, but also can be utilized to improve cultivars through introduction of candidate genes from wild relatives, which are incompatible with cultivated rice species for crossing and hence, inaccessible to breeding programs.

4. CRISPR/Cas gene editing in rice

In rice genome, many untapped genes are yet to be discovered, which can be utilized in favor of resilience to climate change. Sequence-specific nucleases (SSNs) have been used successfully for targeting and modifying several genes. The CRISPR/Cas9 system has been the most promising for its accuracy, efficiency and cost effectiveness to obtain crops with desired traits over others like zinc finger nucleases (ZFNs) and transcriptional activator like effector nucleases (TALENs) [25,58,59]. The CRISPR mediated gene editing was specific, and attained homozygous condition at initial generations, and the mutant stability frequency was high in rice [60]. Various examples of successful application of CRISPR/Cas9 based genome editing in rice have been documented in Table S2.

4.1. Evolving CRISPR/Cas9 tools for genome editing in rice

The CRISPR/Cas9 system has been divided into two distinct classes [61]. The class 1 includes multisubunit effector complexes. The class 2 system that comprises a single multi-domain protein such as SpCas9, is the most commonly employed system for genome engineering [62]. The HNH-like nuclease domain of SpCas9 cleaves the DNA strand complementary to the guide RNA sequence (target strand), while a RuvC-like nuclease domain is responsible for cleaving the non-target strand. Feng et al. [63] first demonstrated the CRISPR/Cas9 mediated genome editing in rice by targeting the rice outermost cell-specific gene5 (*ROC5*), stromal processing peptidase (*SPP*), and young seedling albino (*YSA*) genes. Subsequently, Jiang et al. [6] successfully mutated promoter regions of two rice genes in protoplasts and confirmed the site specific editing through DNA sequencing. Dwarf and albino rice plants were generated by Shan et al. [7] by knocking out the *OsPDS* (phytoenedesaturase) gene. In the next year, they published a stepwise protocol for the selection of target sites, as well as the design and construction of sgRNAs for sequence-specific CRISPR/Cas-mediated mutagenesis [64]. Zhou et al. [65] generated transgene free plants with

large chromosomal segment deletions using constructs with double sgRNAs targeting at each end of the segment followed by genetic crosses. Xie et al. [24] developed a CRISPR/Cas9 system for multiplex genome editing using the concept of endogenous tRNA-processing system. The off-target effect has been exploited for editing of multiple genes of CDPK family [66]. To establish an efficient HDR-mediated gene targeting system in rice, the DNA ligase 4 gene was first disrupted using CRISPR/Cas system and subsequently, the sgRNAs and HDR template were delivered by *Agrobacterium*-mediated transformation. This produced bi-allelic plants for the ALS gene with high frequency [67]. Mutagenesis efficiency showed positive correlation with the expression levels of Cas9, gRNA [68]. Mendelian segregation of T-DNA produces the transgene free mutants from T_1 generation onwards and selection of appropriate homozygous mutants can greatly reduce the off-target effects and enhance the stability of mutants in further generations.

4.1.1. Expansion of the CRISPR targets

The PAM motif acts like a shoehorn that helps the Cas9 RGEN to clasp down and act upon the spacer sequence. However, this can sometimes limit the identification of spacers, particularly for small and AT-rich genes. Nishimasu et al. [69] engineered a SpCas9 variant (SpCas9-NG) that can recognize relaxed NG PAMs. Discovery of another class 2 CRISPR/Cpf1 (also known as Cas12a) system has further broadened the horizon of genome editing, which has shown efficient genome editing ability in human and rice [8,12,13]. The Cpf1-associated CRISPR system does not require the trans-activating RNA (tracrRNA). Therefore, Cpf1 requires only a 42 nt crRNA, while Cas9 uses ~100 nt sgRNA. The Cpf1-crRNA complex needs a short T-rich TTTV (V = A, C, G) PAM. Unlike Cas9, the Cpf1 introduces a staggered double stranded break with 4–5 bases of 5′-overhang [70]. The saCas9 from *S. aureus*, which is ~ 25% smaller in size than spCas9, recognizes an NNGRRT PAM and cleaves the target DNA at high efficiency with a variety of gRNA spacer lengths [14]. More than 20 variants of Cas9 RGNs have been characterized until today with different PAM requirements though only few of these have been tested in plants. Even some of the RGNs have been re-engineered to broaden their PAM specificity [69,71,72]. An SpCas9 variant (xCas9) that can recognize a broad range of PAM sequences including NG, GAA and GAT has been developed by using phage-assisted continuous evolution [73]. However, its specificity to canonical NGG PAM was greatly reduced in rice in contrast to human model [74]. The CasX (Cas12e) is the most recent addition to the CRISPR toolbox that makes staggered double-stranded break at the target site [75].

4.1.2. Base editing: the precision chemistry on the genome and transcriptome

Today, we can even fix a gene by surgically altering a single base in DNA and RNA. The CRISPR/Cas9-based base editor technology enables direct and irreversible conversion of one base pair to another at the target site without the requirement of a DSB or donor template [76–78]. The base editors comprise a catalytically inactive Cas9 nuclease fused to a cytosine deaminase enzyme and in some cases, a DNA glycosylase inhibitor. The deaminase enzyme removes the exocyclic amine of the target cytosine to generate uracil (C→U). Komor et al. [77] fused a catalytically inactive Cas9 (dCas9) to the APOBEC1 cytidine deaminase (CD) to create the Base Editor 1 (BE1). The binding of dCas9 forms a local denaturation resulting in a bubble with single-stranded DNA (ssDNA). The ssDNA specific CD mediates the direct conversion of cytidine to uridine within a window of approximately five nucleotides. Later they improved their base editor by adding a Uracil glycosylase inhibitor (UGI), which impedes uracil excision and a Cas9 nickase (nCas9) targeting the non-edited strand to manipulate the cellular DNA repair response that favored the desired base-editing with minimal (typically ≤1%) INDEL formation. Base editing has been successfully implemented in rice with similar approach [79,80]. Ren et al. [81] produced an improved base editor using a variant of human AID (hAID), another type of cytosine deaminase that showed enhanced base editing efficiency in generating both gain-of-function and loss-of-function mutants of rice. RNA base editors have similar strategy but use components that target RNA [82].

The deamination of cytosine is spontaneous and results in transitions from C•G to T•A base pairs but T•A to C•G conversion is not common. Interestingly, the deamination of adenine yields inosine, which is treated as guanine by polymerases. Adenine base editors (ABEs) are the most novel kind of base editors that mediate the conversion of A•T to G•C in genomic DNA [76]. A tRNA adenosine deaminase, fused to a catalytically impaired Cas9 mutant (dCas9), was employed to operate on DNA to convert targeted A•T base pairs to G•C. The addition of ABEs to the toolbox enables all four transitions (C to T, A to G, T to C, and G to A) in genomic DNA. Base editing is an extremely powerful tool added to molecular breeder's toolbox that can be used to create artificial germplasm diversity for crop plants like rice.

4.2. CRISPR-mediated genome editing for abiotic stress tolerance

An HSA1 (heat-stress sensitive albino 1) deletion mutant generated by CRISPR/Cas9 showed heat sensitivity than the wild-type allele [83]. The role of rice gene OsBBS1 in early leaf senescence and salt stress sensitivity was verified by similar way. Knocking out of the OsAnn3 gene made rice plants susceptible to cold [84]. The genome editing has shown that the OsMIR528 is a positive regulator of salt stress. The regulatory function of the GT-1 element in the salt induction of OsRAV2 was verified in plants with targeted mutations [85]. Low cadmium accumulation in rice without affecting the yield was achieved by gene editing of OsNramp5 [86]. Lou et al. [87] delineated the function of two rice SnRK2 genes, osmotic stress/ABA-activated protein kinases SAPK1 & 2 in salinity tolerance by generating loss-of-function mutants. A large-scale rice mutant library has been constructed using CRISPR/Cas9 system for studying gene functions [88]. Overall, in pursuing goals associated with customized genome editing and chromosome engineering through CRISPR system should be of significant benefit to the rice research and agricultural industries.

5. Challenges

5.1. Off-target effects

One of the most debated criticism of CRISPR technology is the risk of accidentally mutating non-target genes of the manipulated organism that may produce unintended biological effects to its ecosystem. Random mutations may activate some unwanted genes such as disease susceptibility genes. Gene editing may also lead to translocations of chromosomal segments and genome instability. Several strategies have been designed to reduce the off-target risks of Cas9 by optimizing the sgRNA design. Use of truncated sgRNA has been shown to reduce undesired mutagenesis at some off-target sites without sacrificing on-target genome editing efficiencies [89]. Muller et al. [16] reported a *Streptococcus thermophilus* Cas9 with a longer PAM sequence and consequently with less off-target activity. The nmeCas9 system offers another safer alternative for precision genome engineering applications though it has lower efficiency than spCas9 [15]. Use of paired Cas9 nickases, which generate two single-strand breaks (SSBs) or nicks on different DNA strands was shown to be highly specific and effective without off-target activity [90]. The Cas9 off-target activity can be regulated by choosing suitable sgRNA sequence and experimental conditions [91]. The length of gRNA and in vivo concentration of Cas9 enzyme can also be optimized for minimal off-target activity [92]. The off-target mutants can be screened by whole genome sequencing. The GUIDE-Seq technology enables genome-wide unbiased identification of DSBs by Cas9 RGEN [93]. Unintended rearrangements can also be monitored by LAM-HTGTS [94] and Digenome-seq [95]. In case of

plants, mutants free of CRISPR cassette can be backcrossed with the wild type parents to get near isogenic lines with minimal risks. As many other technologies, the CRISPR technology is evolving at a much faster pace to become perfect in near future.

5.2. Transformation efficiency

Another important bottleneck of plant genome engineering is the efficiency and speed of genetic transformation. Though *Agrobacterium*-mediated transformation of rice has gained significant increment in transformation efficiency, the speed still remains a constraint, particularly for larger constructs carrying the Cas9 and gRNA expression cassettes [96,97]. Protoplast genome editing using CRISPR/Cas method is being used as a faster alternative for functional evaluation of genes and characterization of different CRISPR techniques. However, this technique needs to be improved further for high-throughput testing of unknown genes and to generate elite rice cultivars.

6. Prospects and personal perspectives

The molecular breeding in past three decades faced two major bottlenecks. The first concern resulted from the use of marker genes during genetic transformation. Since the genetic transformation efficiency of transgenes was relatively low, researchers used marker genes to select the transformed cells from the mesh of untransformed cells. Most of the marker genes were either an antibiotic or a herbicide tolerance gene, which raised huge protest among social workers and other regulatory bodies. Several strategies have been published to overcome this but still the acceptance of transgenic material remains questionable in most part of the world. The second concern was about the transformation methodology resulting in random insertion of the transgene at any locus along any chromosome. This can potentially disrupt a resident gene or can bring an unwanted phenotype. Another concern was the multicopy and partial integration of the transgene that can result in silencing of the gene expression. The genome engineering can have four major applications in plant molecular breeding in addition to overcoming all of the above challenges. The first and probably the easiest application of CRISPR technology is the gene disruption/knock out using NHEJ. As the recent outburst of NGS data and revolution in computational ability continues to identify and predict novel genes and gene networks, it poses a big challenge to biologists to validate these gene functions *in vivo*. CRISPR mediated gene knock out will help to validate these at the fastest pace to identify genes that significantly affect the plant abiotic stress tolerance. One of the enticing expectation from CRISPR technology is the ability to repair (allelic improvement) or replace (allelic substitution) the mutated or inferior copies of agronomically important genes by HDR. It can be used to create efficient gene knockin or knockout line with point mutations or modified sequence segments. Additionally, it can be used to add a restriction site into the region of interest or even to add a small tag to the gene/protein. The CRISPR/Cas system can also be used to reprogram the expression of the target gene by permanently replacing its promoter or by adding an enhancer to a dCas9 enzyme, designed to bind near the promoter of the target gene. The third application is introduction of a transgene into the host genome at a selected locus or hotspot. Introduction of transgene into selected locus will result in stable expression without disrupting any other gene while the CRISPR/Cas T-DNA can be segregated out (meiotic purification) to generate improved varieties without scars of bacterial genome. The fourth application is to create artificial genetic diversity in rice cultivars by using base editors. Additionally, CRISPR technology can be used for epigenetic modulation and genome imaging.

While the risks of deploying CRISPR may be real, higher are the risks posed by climate change to the food security. Therefore, it will be wise to use CRIPSR technology to develop climate smart rice varieties, capable of withstanding volatile weather, to feed the ever-growing world population.

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Appendix A. Supplementary data

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