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Applications of CRISPR/Cas9 for Biotic and Abiotic Stress Resistance of Rice (*Oryza sativa*)

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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Review Article

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ABSTRACT

Biotechnology has played an important role in the development of varieties with enhanced biotic and abiotic stress tolerance, higher yield potential and an improved resistance towards major diseases and pathogens. The precision of the CRISPR-Cas9 system's target recognition is notably dependable because it employs the Watson and Crick model to identify target sites and utilizes sequence analysis to pinpoint potential off-target locations. In a study carried out to develop resistance against bacterial blight on CO51 variety of rice, a single guide RNA was designed using the structure and sequence information obtained through Ensembl Plants database. The integration of CRISPR/Cas9 technology with traditional breeding methods and other advanced biotechnological tools may further enhance the development of stress-resilient rice varieties with improved productivity and sustainability.

Keywords: Stress-resilient rice varieties; abiotic factors; biotechnology; CRISPR-Cas9.

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1. INTRODUCTION

“Rice is one of the major crops which provides source of food for more than over 3.5 billion people over the world” [1]. “It has been a great source of carbohydrate to the two third of the world population. Hence it has been a rising base for advancements to overcome the degrading factors caused due to environmental and human impacts with the help of biotechnology. Enhancements in the resistance to biotic and abiotic factors has been the most effective and economical way for controlling various diseases, sustainability and improving the quality of the crop. Various major abiotic factors such as drought, cold and salinity and biotic factors such as blast disease, bacterial leaf blight, sheath blight, brown spot, false smut and brown plant hopper are crucial aspects of declining the quality and productivity of rice all over the world” [2]. In past few years various approaches such as molecular, mutational and conventional breeding has been great pioneers for contribution towards the enhanced rice productivity [3]. Biotechnology has played an important role in the development of varieties with enhanced biotic and abiotic stress tolerance, higher yield potential and an improved resistance towards major diseases and pathogens.

“Clustered regularly interspaced short palindromic repeats associated endonuclease Cas9 is the most advanced genome editing tool in plant biology” [4]. “It is biotechnological mechanism consisting of a short RNA molecule called as guide RNA that is associated with a DNA endonuclease called CRISPR-Associated Protein 9 (Cas9). CRISPR/Cas9 is a technique with higher precision and efficiency to achieve the improvement in different plants traits for the agricultural development”[5]. “In this generation of biotechnology CRISPR/ Cas9 is mostly adopted genome editing technology (GET) due to its higher efficiency, simplicity and due to its versatile mechanisms” [6].

“The precision of the CRISPR-Cas9 system's target recognition is notably dependable because it employs the Watson and Crick model to identify target sites and utilizes sequence analysis to pinpoint potential off-target locations” [7]. “In the CRISPR/Cas9 mechanism, foreign DNA is cleaved using two key components which are Cas9 and a single guide RNA (sgRNA). Cas9, which is a DNA endonuclease can be sourced from various bacteria and it consists of two domains which are the HNH domain and the RucV-like domain. The HNH domain cuts the complementary strand of CRISPR RNA (crRNA), while the RucV-like domain cleaves the opposite strand of the double-stranded DNA” [5]. “The sgRNA is an synthetic RNA molecule approximately 100 nucleotides long. Its 5' end contains a 20-nucleotide sequence serving as a guide for identifying the target sequence, accompanied by a protospacer adjacent motif (PAM) sequence, which typically follows the NGG (N representing any nucleotide and G representing guanine) consensus. At the 3' end of the sgRNA, a loop structure facilitates the anchoring of the target sequence through the guide sequence, forming a complex with Cas9. This complex then cleaves the double-stranded DNA, creating a double-strand break (DSB) at the targeted site” [5]. “Following the generation of a DSB, the DNA repair mechanisms of nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) are initiated. In most cases, NHEJ is employed to repair the DSB, which is a straightforward method resulting in mismatches and gene insertions/deletions (InDels), ultimately leading to gene knockout. In the presence of an oligo-template, HDR is capable for facilitating specific gene replacement or the insertion of foreign DNA sequences” [5,8-10].

In consideration of different biotic stresses CRISPR Cas9 has been a great pioneer for the development of varieties which are resistant to Bacterial leaf blight caused by *Xanthomonas oryzae* [11], Blast disease caused by *Magnaporhte oryzae* [12], Rice tungro disease caused by Rice tungro bacilliform virus (RTBV) and Rice tungro spherical virus (RSTV) [13] and some of the major diseases caused by different insect pests. While on the other hand CRISPR Cas9 has been able to achieve significant tolerance over abiotic factors affecting the productivity and quality of rice such as drought stress which is major abiotic stress that limits rice production in rainfed lowland rice agro-systems [14]; salinity stress that is the significant abiotic stress that affects the growth of the plant, it’s development and makes natural production inconvenient [15]; cold stress which can manifest as chilling injury, which occurs when temperatures drop below the threshold for optimal rice growth; heat stress due to high temperatures during the reproductive stage of rice can lead to reduced grain quality and yield losses and flooding stress which is the reason of

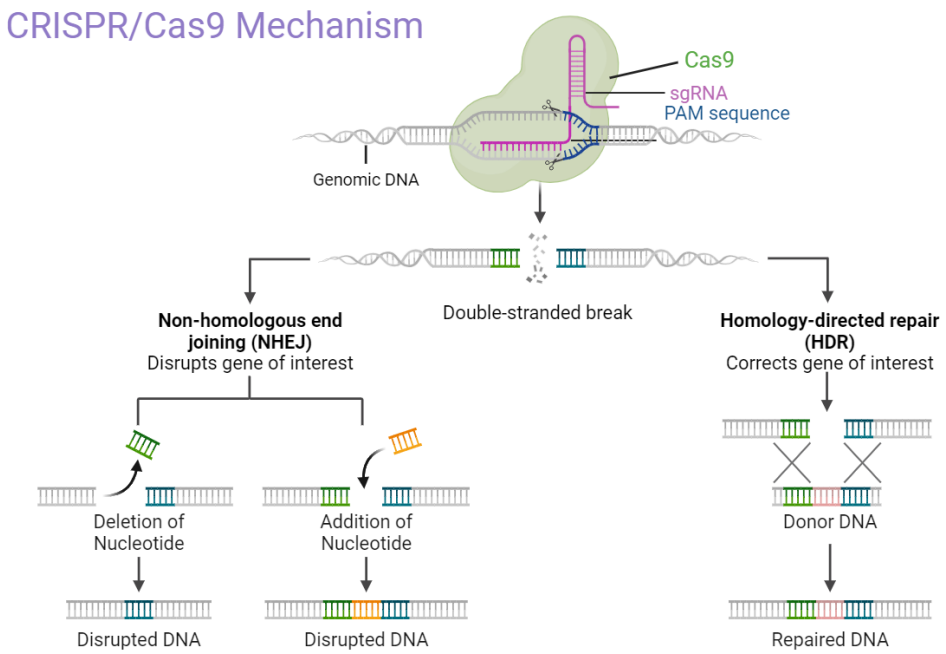

Fig. 1. CRISPR/Cas9 Gene editing mechanism

the oxygen deprivation due to excessive or prolonged flooding which majorly occurs in regions prone to heavy rainfall or seasonal flooding.

2. ROLE OF CRISPR IN BIOTIC STRESS RESISTANT OF RICE

In a study carried out to develop resistance against bacterial blight on CO51 variety of rice, a single guide RNA was designed using the structure and sequence information obtained through Ensembl Plants database. This gRNA was used to target EBE of the promoter region of the *OsSWEET13* gene of the rice. The sgRNA sequence, comprising the upper strand 5'-GCTCAACACCACGAAAATATA-3' and the lower strand 5'-GTATATTTTCGTGGTGTTGAG-3', has been intentionally constructed with compatible adaptors to simplify its insertion into the pRGEB32 binary vector within the exact EBE region to promote cloning and then transferred into LBA4404 *Agrobacterium* strain which was used in transformation experiments [16].

"In order to develop resistance against bacterial blight alterations were made to the *OsSWEET14* gene in the rice variety Zhonghua 11, a CRISPR/Cas9 construct designed to target two specific locations within the coding region of the OsSWEET14 genome sequence was built and then subsequently introduced this construct into the Zhonghua 11 genetic background through transformation process to develop resistance against leaf blight without any penalty"[11].

"For creating a CRISPR/Cas9 system to develop resistance against blast aimed at modifying the *OsERF922* gene in rice, selection of a 20-base pair nucleotide sequence that encompasses the start codon within the open reading frame of OsERF922 was done. This chosen sequence has been designated as the target site ERF922-S2. The anticipated cleavage site for Cas9 within the gene's coding region was situated seven base pairs downstream from the ATG initiation codon. The CRISPR/Cas9 vector served as the foundation for constructing the binary plasmid pC-ERF922"[12].

"To create mutants with resistance to bacterial leaf streak and rice blast, alterations in *Pi21* and *OsSULTR3*;6-EBE, a pair of sgRNAs was devised. These sgRNAs target the second exon of the *Pi21* gene (LOC_Os04g32850) and the promoter region of the *OsSULRT3*;6 gene (LOC_Os01g52130). Two sgRNAs were integrated into the CRISPR/Cas9 vector, and the resulting vector plasmid was employed for the transformation of 58B rice using Agrobacterium tumefaciens EHA105. All 15 transgenic seedlings of 58B exhibited positive traits, achieving a transformation rate of 100%" [17].

"For the construction of CRISPR/Cas9 vectors for conferring rice tungro spherical virus, targeting sequences for three gRNAs within the region spanning nucleotide positions 4140 to 4414 of eIF4G were devised. The selection of target locations was informed by a prior study indicating an association between SNPs at positions 4387 and 4390 and the response to RTSV" [18]. "gRNA1 was crafted to target approximately 230 base pairs upstream of the specified SNPs, gRNA2 was tailored to target 10 base pairs upstream of this region, and gRNA3 was devised to target 5 base pairs downstream of SNP4390. The T-DNA within the final vectors, namely pCas9-eIF4G-gRNA1, pCas9-eIF4G-gRNA2, and pCas9-eIF4G-gRNA3, comprised three cassettes: (i) the Cas9 cassette, facilitating the expression of a plant codon-optimized Cas9 from Streptococcus pyogenes under the control of the Zea mays ubiquitin (ZmUBI1) promoter, (ii) the gRNA cassette, responsible for the expression of one of the three gRNAs, each driven by the Triticum aestivum U6 promoter, and (iii) the plant selection cassette containing hygromycin resistance (hyg) driven by the CaMV35S promoter" [13].

"For the development of resistance against blast in an *indica* TGMS rice line Longke638S use of CRISPR/Cas9-mediated gene editing was done to rapidly install mutations in three known broad-spectrum blast-resistant genes, *Bsr-d1, Pi21 and ERF922*. Targeted mutagenesis of *Bsr-d1, Pi21* and *ERF922* genes, three sgRNAs were designed. A constructed expression vector featured the Cas9 cassette driven by the ubiquitin promoter along with a single guide RNA (sgRNA) scaffold governed by the rice small nuclear RNA promoters. This vector was used to target one or multiple sites via single construct, to create bsr-d1- or pi21- or erf922-targeted single gene mutants and triple bsr-d1/pi21/erf922 mutants. The transformed expression vectors were employed for the introduction of genetic material into the TGMS variety Longke638S (LK638S) through Agrobacterium-mediated transformation. 34 rice blast resistance lines were obtained based on hygromycin resistant selection for bsr-d1, 22 for pi21, 45b for erf922 and 35 for triple bsr-d1/pi21/erf922" [19].

To generate resistance to bacterial leaf blight of rice an effort was undertaken to induce targeted insertions or deletions (InDels) in the Effector Binding Element (EBE) of the *SWEET13* gene in CO51. A suitable sgRNA sequence was devised, inserted into the widely used binary vector, pRGEB32, which carries the Cas9 gene. This vector was then transferred into the Agrobacterium strain, LBA4404, and utilized in transformation experiments. Agrobacterium strain carrying the recombinant pRGEB32, which contains the *SWEET13* sgRNA expression cassette, was used to infect immature embryos of CO51. Calli that underwent co-cultivation were exposed to hygromycin selection to encourage the preferential growth of transformed cells and facilitate the retrieval of edited plants. Thirteen plants, originating from four distinct transgenic events, were produced with a transformation efficiency of 1.6 percent [16].

To achieve resistance against Rice Tungro disease developed due to interaction between rice tungro spherical virus and rice tungro bacilliform virus twenty-four groups consisting of 1680 immature embryos underwent co-cultivation with the Agrobacterium strain LBA4404 containing the pRGEB32-eIF4G construct. The embryos subjected to co-cultivation were subsequently cultivated on hygromycin selection. Upon transfer to regeneration medium, hygromycin-resistant calli lines progressed into fully developed plants. A total of thirteen plants were produced with a transformation efficiency of 0.8%. All thirteen lines tested positive for PCR amplification of hpt and cas9 genes [20].

Table 1. List of Genes targeted by CRISPR Cas9 for biotic stress resistance

Rice Variety	Target Gene	Molecular Function	References
CO51	*OsSWEET13*	Enhanced resistance to bacterial blight	16
Zhonghua 11	*OsSWEET14*	Enhanced resistance to bacterial blight	11
Kuiku131	*OsERF922*	Enhanced resistance to blast	12
58B	*Pi21*	Enhanced resistance to bacterial leaf streak	17
58B	*OsSULRT3*	Enhanced resistance to blast	17
Longke638S	*Bsr-d1, Pi21 and ERF922*	Enhanced resistance to blast	19
CO51	*SWEET13*	Enhanced resistance to bacterial leaf blight	16

3. ROLE OF CRISPR IN ABIOTIC STRESS RESISTANT OF RICE

"For the construction of gene-edited rice having tolerance to drought and salinity by using CRISPR/Cas9 technology, an sgRNA was designed targeting the second exon of OsPUB7. The chosen sgRNAs exhibited a 50% GC content, an out-of-frame score of 84.7, and were devoid of any mismatches in the genome, aiming to minimize the probability of off-target effects. The chosen sgRNA was situated approximately 113 base pairs from the 5'-end of the second exon and was evaluated as a target within this specific region. A Ti-plasmid vector designed for plant gene editing was created, featuring Cas9 regulated by the 35S promoter, sgRNA regulated by the OsU3 promoter, and the bar gene serving as the selection marker. The entire vector, designated CRISPR/Cas9-OsPUB7, was introduced into the callus derived from the seeds of the wild-type (WT) plant (Dongjin) through an Agrobacterium-mediated technique, resulting in the acquisition of 31 transformants" [21].

"In order to develop novel rice germplasm for salt tolerance at seedling stage using CRISPR-Cas9, the entire nucleotide sequences of the OsRR22 gene (Os06g0183100) were retrieved from the NCBI database, and specific primer target sites were designed within the exon region. The kit facilitated the swift and straightforward insertion of the gRNA target sequence into the Cas9/gRNA plasmid. The resulting Cas9/gRNA plasmid was capable of concurrently expressing the plant codon-optimized Cas9 protein and gRNA. The target gene was subjected to knockout and editing utilizing CRISPR technology" [22].

"Utilizing CRISPR/Cas9 in response to drought stress of rice three CRISPR/Cas9 constructs targeting OsERA1, each containing a specific gRNA, were employed for the transformation of the rice cultivar Nipponbare. Nipponbare is commonly utilized as a standard cultivar in lowland rice studies. They produced 7, 201 and 65 T0 transformants for the constructs with gRNA1, gRNA2, and gRNA3, respectively. Multiple T0 transformants with target mutations for all three constructs were identified through PCR-based genotyping" [23].

For the purpose of creating a mutation that specifically targets the OsRR22 gene in rice in response to salinity stress, a 20-base pair nucleotide sequence within the first exon of OsRR22 was selected as the target site. The binary plasmid Cas9-OsRR22-gRNA was then constructed, utilizing the CRISPR/Cas9 vector described earlier by Ma et al. [24]. "The vector was employed for the transformation of the rice variety WPB106 through Agrobacterium-mediated transformation. Through site-specific PCR and Sanger sequencing, a collective of nine WPB106 mutants were obtained from 14 T0 hygromycin-resistant transgenic WPB106 plants, constituting a success rate of 64.3%" [25].

"To develop novel salinity-stress tolerant hybrid rice, genome editing through CRISPR/Cas9 was utilized to create mutagenesis lines in OsRR22. Three gRNAs designed for the 22KT1, 22KT2, and 22T3 target sites were employed to target the third exon of OsRR22. These sites were selected due to their proximity to a single nucleotide polymorphism (SNP) in the third exon of OsRR22, a variant known to be linked with enhanced salinity tolerance. Two distinct CRISPR/Cas9 vectors were constructed, one comprising two gRNAs (pC-22-KT12), and the other containing a single gRNA (pC-22-T3). These vectors were introduced into 733B and HZ varieties through Agrobacterium-mediated transformation" [26].

"In pursuit of rice mutants characterized by both high yield and robust cold tolerance, three genes were selected for editing: OsPIN5b, associated with panicle length; GS3, linked to grain size; and OsMYB30, involved in cold tolerance. Two target sites were chosen for each gene: OsPIN5b-site1 and OsPIN5b-site2, GS3-site1 and GS3-site2, and OsMYB30-site1 and OsMYB30-site2. The Golden Gate assembly method was employed to insert six sgRNA cassettes into the CRISPR/Cas9 vector. Through the analysis of PCR products from the target sites, a cumulative of thirty-eight distinct transgenic lines were generated. The outcomes indicated a notable editing efficiency of the CRISPR/Cas9 system in the T0 generation: 53% for OsPIN5b-site1, 42% for OsPIN5b-site2, 66% for GS3-site1, 63% for GS3-site2, 63% for OsMYB30-site1, and 58% for OsMYB30-site2" [27].

To assess the function of OsSAP gene in drought conditions, GeD0 (Genome-editing Drought gene OsSAP 0 generation) lines were developed by editing the OsSAP gene using CRISPR/Cas9. Within the CRISPR/Cas9 vector designed for rice expression, the guide RNA for OsSAP is controlled by the U3 promoter. The guide RNA is integrated into the *BsaI* site of the pRGEB32

Table 1. List of genes targeted by CRISPR Cas9 for abiotic stress resistance

Rice Variety	Target Gene	Molecular Function	References
Dongjin	*OsPUB7*	Enhanced tolerance to drought and salinity stress	21
R192	OsRR22	Enhanced tolerance to salinity stress	22
Nipponbare	OsERA1	Enhanced tolerance to drought stress	23
WPB106	OsRR22	Enhanced tolerance to salinity stress	25
733B	OsRR22	Enhanced tolerance to salinity stress	26
Nipponbare	OsPIN5b, GS3, OsMYB30	Enhanced tolerance to cold stress	27
Ilmi	OsSAP	Enhanced tolerance to drought stress	28
Nipponbare	OsNAC050	Enhanced tolerance to cold stress	30
Taipei309	*OsAnn3*	Enhanced tolerance to cold stress	31

vector. Three guide RNAs were developed, and their GC contents were adjusted to fall within the range of 50–70%. Subsequently, the CRISPR/Cas9 vector, with the integrated sgRNA, was introduced into the callus using Agrobacterium medium. The results indicated that OsSAP plays a crucial role in promoting tolerance to drought stress, is associated with leaf senescence, and holds promise as a potential gene for enhancing yield in drought-stress conditions during the development of new rice cultivars [28].

To enhance cold tolerance in Nipponbare variety of rice mutants of OsNAC050 were created through the CRISPR-Cas9 knockout vector pZHY988. CRISPR-P was employed for sgRNA (small guide RNA) design and to anticipate potential off-target sites. Following the synthesis of the sgRNA, the vector was assembled using recombinant DNA techniques. Once the desired vector was confirmed through detection and DNA sequencing, the rice callus underwent transformation using Agrobacterium tumefaciens EHA105 [29]. The OsNAC050 mutant lines in rice exhibited enhanced capability in managing reactive oxygen species (ROS) induced by low-temperature stress. This aligns with the observed heightened tolerance to low temperatures in the mutant plants [30].

To assess the expression of *OsAnn3* gene for cold stress in rice quantitative reverse-transcription (qRT)-PCR was employed to assess the expression of the annexin gene OsAnn3 in leaf tissue of the wild-type rice variety Taipei309. This evaluation was conducted under normal conditions (28°C) and following a 3-day treatment at 4~6°C. The findings revealed a 9.8-fold up-regulation in the transcript levels of OsAnn3 in Taipei309 after exposure to cold treatment, in comparison to the levels observed under normal conditions. Hence, it was proposed that OsAnn3 underwent regulation in response to cold stresses, potentially playing a role in enhancing cold tolerance in rice [31].

4. CONCLUSION

The studies demonstrate the remarkable potential of the CRISPR/Cas9 system as a precise and efficient genome-editing tool for enhancing biotic and abiotic stress tolerance in rice. Through targeted mutagenesis, researchers have successfully developed rice varieties with improved resistance to major biotic stresses such as bacterial blight by editing genes like OsSWEET13 and OsSWEET14, blast disease by modifying OsERF922 and Pi21, and rice tungro disease by targeting eIF4G. Additionally, CRISPR/Cas9 has facilitated the creation of rice mutants with elevated tolerance to critical abiotic stresses, including drought stress via OsERA1 and OsSAP gene editing, salinity stress through modifications in OsPUB7 and OsRR22, and cold stress by targeting genes like OsPIN5b, GS3, OsMYB30, OsNAC050, and OsAnn3.

The studies highlight the remarkable precision and efficiency of the CRISPR/Cas9 system in introducing targeted mutations in specific genes associated with stress response pathways, without compromising yield or other agronomic traits. This technology has accelerated the development of stress-tolerant rice varieties, which is crucial for ensuring food security in the face of environmental challenges and climate change. However, the studies also acknowledge the need for further research to evaluate the long-term stability and potential off-target effects of CRISPR/Cas9-mediated modifications in rice. Additionally, the integration of CRISPR/Cas9 technology with traditional breeding methods and other advanced biotechnological tools may further enhance the development of stress-

resilient rice varieties with improved productivity and sustainability.

Overall, the CRISPR/Cas9 system has emerged as a game-changer in rice improvement, offering unprecedented opportunities for precise genome editing and the development of stress-tolerant rice varieties with enhanced resilience and productivity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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