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REVIEW ARTICLE

Application of CRISPR/Cas9 Genome Editing System in Cereal Crops

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Abstract: Recent developments in targeted genome editing accelerated genetic research and opened new potentials to improve the crops for better yields and quality. Genome editing techniques like Zinc Finger Nucleases (ZFN) and Transcription Activator-Like Effector Nucleases (TALENs) have been accustomed to target any gene of interest. However, these systems have some drawbacks as they are very expensive and time consuming with labor-intensive protein construction protocol. A new era of genome editing technology has a user-friendly tool which is termed as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated protein9 (Cas9), is an RNA based genome editing system involving a simple and cost-effective design of constructs. CRISPR/Cas9 system has been successfully applied in diverse crops for various genome editing approaches. In this review, we highlight the application of the CRISPR/Cas9 system in cereal crops including rice, wheat, maize, and sorghum to improve these crops for better yield and quality. Since cereal crops supply a major source of food to world populations, their improvement using recent genome editing tools like CRISPR/Cas9 is timely and crucial. The genome editing of cereal crops using the CRISPR/Cas9 system would help to overcome the adverse effects of agriculture and may aid in conserving food security in developing countries.

Keywords: CRISPR/Cas9 system, Genome editing, Rice, Wheat, Maize, Cereal.

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1. INTRODUCTION

Generating targeted genetic changes in crop plants is one of the key requirements for improving them for many useful traits. The plant biotechnology field is now harnessing genome editing technologies to edit specific genomic sequences of crop plants. Such methods rely on Sequence-Specific Nucleases (SSNs) to introduce Double-Stranded Breaks (DSBs) or single-stranded breaks at a targeted location in the genome. Repair of DSBs is predominantly done through two major pathways such as Non-Homologous End-Joining (NHEJ) repair, which ends up in insertions or deletions and Homology-Directed Repair (HDR) that carries out precise genomic changes [1, 2]. Early SSNs, like Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), were successfully utilized in plants for genome modifications [3, 4]. ZFNs and TALENs rely on protein DNA interactions to recognize specific DNA sequences; however, these techniques have distinctive limitations and proved difficult in plasmid construction and are also very expensive [5, 6].

Recently, a new genome editing technology referred to as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated protein9 (Cas9) system emerged as a popular tool and successfully demonstrated in diverse systems and it also offers novel alternatives in basic plant science and crop improvement studies [7, 8]. CRISPR/Cas9 system is now widely adopted and applied in many plants including *Arabidopsis thaliana* [9, 10], rice [11], wheat [11], and tobacco [10, 12]. The CRISPR/Cas9 system also enhanced hybrid-breeding techniques, allowing agricultural crops to be modified, even in a single generation. As a result, the CRISPR/Cas9 system has been adopted for the rapid improvement of agricultural crops. In this mini-review, we discuss the application of the CRISPR/Cas9 system in various cereal crops. We list the details on the gene(s) targeted, plasmids used, method of transformation and frequency of mutations obtained using CRISPR/Cas9 in cereal crops.

2. CRISPR/CAS9 SYSTEM

The CRISPR/Cas9 system is a prokaryotic RNA-mediated adaptive immune system in bacteria and archaea that holds a defense against phages and other foreign genetic elements. The CRISPR/Cas system is divided into two classes (1 & 2). Each class is subdivided into three types. Each class contains 3 subtypes (Class 1; type I, III, and IV and Class 2; type II, V, and VI). Type I contains eight different Cas operons; type II contains four Cas operons and trans-activating CRISPR RNA: CRISPR RNA (tracRNA: crRNA); type III contains eight Cas operons and Csm/Cmr complexes; type IV contains two Cas operons and four DinG/Csf proteins; type V contains four Cas

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operons and four Cpf2 proteins; and type VI contains three Cas operons and three C2c2 proteins. Even though other CRISPR/Cas systems have numerous Cas operons, the type II CRISPR/Cas system composing of Cas9 protein has been utilized as a simple programmable genome editing tool [13].

The type II CRISPR/Cas system adopted from *Streptococcus pyogenes* has been widely used as a CRISPR/Cas9 genome editing tool [14]. The type II CRISPR/Cas9 construction requires only synthetic "linker loop or scaffold" that fuses the protospacer-containing crRNA and tracRNA into single guide RNA (sgRNA). The sgRNA forms complex with Cas9 to the target DNA sequence and initiate DSBs at the 3 nucleotides (nt) downstream from the Protospacer Adjacent Motif (PAM) sequence [14]. The CRISPR/Cas9 generated DSBs are filled either through NHEJ or HDR strategy.

3. APPLICATION OF CRISPR/CAS9 SYSTEM GENOME EDITING IN CEREAL CROPS

Agriculture is the key sector of the planet to sustain human food. Currently, crop production is facing numerous challenges collectively due to climatic change, various abiotic stresses (including drought), and damage by pathogens. To overcome these challenges, plant scientists have applied several novel molecular tools to improve the quantity and quality of yield. Recently, the CRISPR/Cas9 genome editing system has been applied in many crops to improve stress tolerance and to increase the yield [7, 15].

Cereal crops are stable foods primarily supplying energy and nutrients for thousands of years and have contended a necessary role for human life. Cereal crops have been widely introduced into cultivation in greater quantities due to the supply of 90% of food to the global population than other crops. Mainly rice, wheat, and maize are the major stable cereals to the majority of the world population. But, worldwide threats like heat, drought, salinity, frost, bacteria, flora, virus, *etc*. are inflicting serious suffering to cereal crops [16]. So, to overcome these challenges, several new and novel molecular tools are being utilized for improving the cereal crops. Newly discovered CRISPR/Cas9 system has the potential to improve the cereal crops for withstanding adverse climatic conditions. So, in this mini-review, the details on the application of the CRISPR/Cas9 system in cereal crops are discussed (Table **1**).

3.1. Rice

Rice is a staple food on which one half of the global population depend upon. Rice is employed as a model crop for monocotyledon plants due to its small genome size with an early release of the whole genome sequence. Several genome engineering studies have been demonstrated and more recently, the CRISPR/Cas9 genome editing tool has been utilized in editing the genome of rice (Table **1**). The CRISPR system has been successfully applied in rice using codon-optimized *sp*Cas9 by targeting the *phytoenedesaturase* (*OsPDS*) gene [11]. To disrupt this gene, two sgRNAs (SP1 & SP2) were designed and observed 15% mutations in protoplasts and 9% mutations in transgenic lines [11]. Similarly, the *mitogen-activated Protein Kinase5* (*OsMPK5*) gene of rice was knocked-out using the CRISPR/Cas9 system to enhance disease resistance in rice. They observed a 3-8% mutation in rice protoplasts [17]. Multiplex genome editing also approached using CRISPR/Cas9 systems in rice [18]. In this study, the authors engineered multiple sgRNAs to express under the *U3/U6* promoter and confirmed that the multiplex genome editing is possible in rice [18]. Hu *et al*. (2016) demonstrated genome editing using the Cas9-VQR variant in rice. They selected a *narrow leaf1* (*NAL1*) gene and designed two sgRNAs to target this gene but the editing efficiency was low [19]. Later, the same group used different promoters of rice *UBIQUITIN1 (UQ1)* and *ACTIN1 (ACT1)* in the CRISPR/Cas9-VQR system that shows high editing potency [20]. Additionally, to achieve high genome editing efficiency in rice, more specific Cas9 variants, *sp*Cas9 (1.0), *sp*Cas9 (1.1), and *sp*Cas9-high-fidelity variant 1 (HF1) VQR were used and this helped to achieve high target efficiency [20]. Recently, to boost the salt tolerance in rice, the authors knocked-out the *O. sativa response regulator 22* (*OsRR22*) gene using the Cas9-*OsRR22*-gRNA expression vector and achieved 64.3% mutation in T0 lines, this knockout in *OsRR22* gene using the CRISPR/Cas9 system improved the tolerance to salinity [21]. Many other studies have also been attempted in rice for CRISPR/Cas9-mediated genome editing (Table **1**). Studies like these proved that CRISPR/Cas9 could be successfully exploited for improving the tolerance of rice to stresses like salinity.

3.2. Wheat

CRISPR/Cas9 system has been successfully demonstrated by the knocking-out of *mildew-resistance locus* (*TaMLO*) gene in wheat [22]. The knock-out mutation frequency of the *TaMLO* gene was 28.5% which results in improved disease resistance in wheat [22]. This initial successful knock-out in wheat brings the importance of the CRISPR/Cas9 system for agriculturally important traits. Similarly, researchers knocked-out *enhanced disease resistance 1* (*TaEDR1*) gene of wheat which is a negative regulator of powdery mildew resistance [23]. Another group targeted *lipoxygenase* genes (*TaLpx1* and *TaLox2*) [22]. Editing of *TaLpx1* & *TaLox2* genes of wheat showed 9 and 45% mutations, respectively [22]. To extend grain size and yield, *TaGASR7*, *TaNAC2*, *TaGW2*, and *TaDEP1* genes of wheat were edited and knocked-out using the CRISPR/Cas9 system resulting in augmented grain weight (27.7%), grain area (17.0%), grain length (6.1%), and grain width (10.9%) on comparison to the wild plants [24]. These studies illustrate the targeted genome editing using CRISPR/Cas9 in wheat to improve the yields and to overcome the adverse conditions in wheat.

3.3. Maize

Maize is one of the most important cereal crops grown under varied environmental conditions. It is one of the third important crops after rice and wheat. Liang *et al.* (2014) first initiated gene knockout in maize using the CRISPR/Cas9 system. They targeted the *ZmIPK* gene of maize that regulates in phytic acid synthesis. They designed two gRNAs to target the respective gene which resulted in 16 to 19% mutation frequency and concluded that the CRISPR/Cas9 is a highly efficient system for gene modification in maize [25]. Similarly,

another group knocked-out the *phytoene synthase* (*PSY1*) gene using sgRNA under the expression of the *U6* promoter [26]. They observed 10.67% cleavage efficiency of the *PSY1* gene

Table 1. Application of CRISPR/Cas9 based genome editing system in cereal crops. Details on the name of the cereal crop, type of study, name of the promoter, method of transformation, and name of the gene-edited are added with respective references.

Name of the Plant	Type of Study	Cas9 Promoter	sgRNA Promoter	Method of Delivery	Target Gene	References
Rice	Functional genomics	*CaMV 35S*	*U6*	*Agrobacterium*-mediated	*OsSWEET14* and *OsSWEET11*	[31]
	Site-directed mutagenesis	*CaMV 35S*	*OsU6-2*	*Agrobacterium*-mediated	*OsMYB1*	[32]
	Site-directed mutagenesis	*CaMV 35S*	*U3*	*Agrobacterium*-mediated	*OsPDS, OsBADH*, *Os02g23823* and *OsMPK2*	[22]
	Site-directed mutagenesis	*CaMV 35S*	*U3 or U6*	Protoplast transformation	*MPK5*	[17]
	Site-directed mutagenesis	*CaMV 35S*	*OsU6-2*	*Agrobacterium*-mediated	*ROC5, SPP* and *YSA*	[34]
	Gene editing	*CaMV 35S*	*U6*	*Agrobacterium*-mediated	*OsPDS, OsPMS3, OsEPSPS, OsDERF1, OsMSH1, OsMYB5, OsMYB1, OsROC5, OsSPP* and *OsYSA***	[35]
	Genome editing	*2 × 35S*	*U6*	*Agrobacterium*- mediated	*TaLOX2*	[22]
	Multiplex editing capability with the endogenous tRNA	*UBI*	*OsU3*	*Agrobacterium*- mediated	*OsMPKs*	[33]
	Multiplex genome editing in monocot and dicot plants	*UBI/35S*	*OsU3/U6*	*Agrobacterium*- mediated	*46 genomic targets*	[18]
	Deletions and heritable small genetic changes induced	*UBI*	*OsU6*	*Agrobacterium*- mediated	*OsSWEET11*, and *OsSWEET14*	[36]
	Functional studies	*OsUBI*	*OsU6, OsU3, and TaU3*	*Agrobacterium*-mediated	*GW2, GW5*, and *TGW6*	[37]
	Functional studies	*35S*	*U3*	*Agrobacterium*-mediated	*OsCYP97A4, OsDSM2, OsCCD4a, OsCCD4b*, and *OsCCD7*	[38]
	Knock-out	*ZmUBI*	*U6*	*Agrobacterium*-mediated	*Gn1a, DEP1, GS3,* and GLW2	[39]
	Functional genomics	*CaMV 35S*	*U6*	*Agrobacterium*-mediated	*OsRAV2*	[40]
	Knock-out	*ZmUBI*	*U3*	*Agrobacterium*-mediated	*ALS*	[41]
	Knock-out	*pHUN411–C3C5*	*U3*	Protoplast transformation	*EPSPS*	[42]
	Knock-out	*ZmUBI*	*U3 or U6*	*Agrobacterium*-mediated	*TMS5*	[36]
	Functional genomics	*2 × P35S*	*U6*	*Agrobacterium*-mediated	*ALS*	[41]
	Knock-out	*ZmUBI*	*U6*	*Agrobacterium*-mediated	*OsERF922*	[43]
	Knock-out	*pCXUN*	*U3*	*Agrobacterium*-mediated	*SBEI* and *SBEIIb*	[44]
	Knock-out	*ZmUBI*	*U6, U3*	*Agrobacterium*-mediated	*OsNramp5*	[45]
	Functional studies	*CaMV 35S*	*U6*	*Agrobacterium*-mediated	*OsAnn3*	[46]
	Knock-out	*OsUBI*	*U3*	*Agrobacterium*-mediated	*OsCCD7*	[47]
	Knock-out	*pZH*	*U3 and U6*	*Agrobacterium*-mediated	*OsFAD2-1*	[48]
	Knock-out	*UBI-1*	*U6*	*Agrobacterium*-mediated	*OsACC-T1OsALS-T1, OsCDC48-T3, OsDEP1-T1, OsDEP1-T2*, and *OsNRT1.1B-T1*	[49]
	Knock-out	*ZmUBI*	*U6*	*Agrobacterium*-mediated	*GS3*, and *Gn1a*	[50]
	Functional studies	*CaMV 35S*	*U3*	*Agrobacterium*-mediated	*GS9*	[51]
	Functional studies	*35S*	*U6*	*Agrobacterium*-mediated	*Bsrk1*	[52]
	Knock-out	*ZmUBI*	*U6*	*Agrobacterium*-mediated	*SAPK2*	[53]
	Knock-out	*ZmUBI*	*U6*	*Agrobacterium*-mediated	*elF4G*	[54]
	Knock-out	*CaMV 35S*	*U6*	*Agrobacterium*-mediated	*Waxy*	[55]
	Knock-out	*PUBI-H*	*U6*	*Agrobacterium*-mediated	*OsRR22*	[56]
	Knock-out	*CaMV 35S*	*U6*	*Agrobacterium*-mediated	*Wx*	[57]
	Knock-out	*CaMV 35S*	*U6*	*Agrobacterium*-mediated	*ISA1*	[58]
Wheat	Site-directed mutagenesis	*CaMV 35S*	*U6*	*Agrobacterium*-mediated	*Inox* and *PDS*	[59]

(Table 1) contd.....

Name of the Plant	Type of Study	Cas9 Promoter	sgRNA Promoter	Method of Delivery	Target Gene	References
	Site-directed mutagenesis	*CaMV 35S*	*U6*	Protoplast transformation	*MLO*	[11]
	Site-directed mutagenesis	*CaMV 35S*	*U3 or U6*	Particle bombardment	*TaMLO-A1*, *TaMLO-B1* and *TaMLO-D1*	[60]
	Genome editing in wheat through transient expression	*UBI*	*TaU6*	Particle bombardment	*immature embryos*	[61]
	Knock-out	-	-	Protoplast transformation	*TaGW2(A1, -B1* and *D1)*	[62]
	Gene editing	*35S*	*U6*	Biolistic	*TaGASR7*	[63]
	Functional genomics	*UBI*	*U6 and U3*	*Agrobacterium*-mediated	*TaPDS*	[64]
	Functional genomics	-	*TaU6*	*Agrobacterium*-mediated	*TaDREB2* and *TaERF3*	[65]
	Knock-out	*2×35S*	*U6*	Biolostic	*TdGASR7*	[66]
	Genome editing	*ZmUBI*	*U6*	*Agrobacterium*-mediated	*TaCKX2-1, TaGLW7, TaGW2*, and *TaGW8*	[67]
Maize	Targeted mutagenesis	*CaMV 35S*	*ZmU3*	*Agrobacterium*-mediated	*ZmIPK*	[25]
	Targeted mutagenesis	*UBI*	*ZmU6*	*Agrobacterium*-mediated	*ZmLIG1*, *ZmM26*, *Zm45*, and *ZmALS1*	[68]
	Targeted mutagenesis	*UBI*	*U6*	Particle bombardment	*ALS2*	[69]
	Genome editing	*2×35S*	*U3*	*Agrobacterium*-mediated	*Zmzb7*	[27]
	Gene editing	*UBI*	*U6*	*Agrobacterium*-mediated	*MYBR* and *AP2*	[70]
	Genetic association	*GOS2*	*GOS2*	Particle-bombarded	*ARGOS8*	[71]
	Targeted mutagenesis	*UBI*	*ZmU6*	*Agrobacterium*-mediated	*Argonaute 18*	[72]
	Knock-out	-	*U3*	*Agrobacterium*-mediated	*MS8*	[28]
	Knock-out	*35S*	*U3*	*Agrobacterium*-mediated	*zyp1*	[73]
	Knock-out	*ZmUBI*	*U6*	*Agrobacterium*-mediated	*ZmLG1*	[74]
	Knock-out	*UBI*	*U6*	Particle bombardment	*SDN1*	[75]
	Targeted mutagenesis	*UBI*	*U3 and U6*	*Agrobacterium*-mediated	*20 genes*	[75]
	Gene editing	*35SPPDK*	*U6*	*Agrobacterium*-mediated	*immature embryos*	[76]
Sorghum	Functional genomics	*Rice Actin 1*	*U6*	*Agrobacterium*-mediated	*DsRED2*	[31]
	Gene editing	*ZmUBI*	*U3*	*Agrobacterium*-mediated	*k1C*	[77]
	Knock-out	*ZmUBI*	*U3*	*Agrobacterium*-mediated	*PMI*	[78]
	Gene editing	*ZmUBI*	*U3*	Particle bombardment	*CAD* and *PDS*	[79]
Barley	Knock-out	*ZmUBI*	*U6*	*Agrobacterium*-mediated	*HvPM19*	[80]
	Gene editing	*ZmUBI*	*U6*	*Agrobacterium*-mediated	*hpt*	[81]
	Fragment Deletions and Small Indels	*ZmUBI*	*U6*	*Agrobacterium*-mediated	*ENGase*	[29]
	Multiplex genome editing	*ZmUBI*	*U6*	*Agrobacterium*-mediated	*HvCKX1*	[82]
	Knock-out	*ZmUBI*	*U3*	*Agrobacterium*-mediated	*HvMORC1*	[30]
	Gene editing	*CaMV 35s*	*U6*	*Agrobacterium*-mediated	*PDS1*	[83]
	Functional studies	*ZmUBI*	-	*Agrobacterium*-mediated	*dsRED*	[84]
	Knock-out	*ZmUBI*	*U6*	*Agrobacterium*-mediated	*hptII*	[85]

Abbreviations used: *Ago; Argonaute, ALS; Aceto Lactate Synthase, Ann; Annexin, AP; Apetala, BADH; Betaine aldehyde dehydrogenase, CAD; Cinnamyl Alcohol Dehydrogenase, CCD; Carotenoid Cleavage Dioxygenase, CKX; Cytokinin oxidase, DREB; Dehydration-Responsive Element-Binding protein, DsRED; Red fluorescent protein, ENGase; Cytosolic endo-beta-N-acetyl glucosaminidase, EPSPS; Enolpyruvylshikimate-3-phosphate, ERF; Ethylene-responsive transcription factor, FAD; Fatty acid desaturase, GASR; GA-induced protein, Gn; Guanine-nucleotide, GW; E3 ubiquitin-protein ligase, Hv; Hordeum vulgare, Hpt; Homogentisatephytyltransferase, Hpt; Hygromycin phosphotransferase, Inox; Inositol oxygenase, IPK; Inositol polyphosphate multi kinase, ISA; Iso amylase, k1C; Alpha-Kafirin, LOX; lipoxygenase, MLO; Mildew resistance locus, MORC; Microrchidia, MPK; Mitogen activated Protein Kinases, MS; Male Sterility, MYB; Transcription factor MYB, Nramp; Metal transporter Nramp, Os; Oryza sativa, PDS; phytoenedesaturase, PM; Protein Membrane, PMI; Phosphor Mannose Isomerase, RAV; Transcription repressor RAV,ROC; Rice outermost cell-specific gene,RR22; Two-component response regulator, SAPK; Serine/threonine-protein kinase, SBE; Starch branching enzyme, SDN; Small RNA degrading nuclease, SPP; Stromal Processing Peptidase, SWEET; Bacterial blight susceptibility genes, Ta; Triticum aestivum, TMS; Thermo-sensitive genic Male Sterility, YSA; Young Seedling Albino, Zm; Zea mays.*

in maize using the CRISPR/Cas9 system. Additionally, they sequenced the mutated gene to verify the mutation efficiency [26]. Targeting the *albino marker* (*Zmzb7*) gene using the CRISPR/Cas9 system results in a 31% mutation frequency in T0 lines [27]. Next, the *thermosensitive genic male-sterile 5* (*ZmTMS5*) gene of maize was targeted using the CRISPR/Cas9 system. The authors designed three sgRNAs to target the *ZmTMS5* gene and generated mutations in protoplasts [28].The edited plants showed biallelic modification which indicates that the CRISPR/Cas9 system has a great potential for targeted mutagenesis for improving the traits in maize [28]. These studies demonstrate that the application of the CRISPR/Cas9 system would advance the breeding approaches in maize and may help for crop improvement.

4. CRISPR/CAS9 GENOME SYSTEM IN OTHER CEREALS

CRISPR/Cas9 system has also been applied in other cereal crops (Table **1**). CRISPR/Cas9 system was attempted in barley by knocking-out the *endo-N-acetyl-b-D-glucosaminidase* (*EN-Gase*) gene [29]. The authors designed five sgRNAs and demonstrated a 78% mutation frequency in T0 and T1 lines of barley. But the transgenic barley plants with frame-shift mutations did not show any difference in phenotype while comparing with the wild plants. From this result, the authors revealed that the CRISPR/Cas9 system has a great potential to knock-out various genes and to understand their functions [29]. Next, to study the function of *MORC* proteins of cereals, researchers used CRISPR/Cas9 knock-out strategy in the *microrchidia (HvMORC1)* gene of barley to check its functions [30]. They generated sgRNA under the *OsU3* promoter and detected a high frequency of mutations. In T0 generations, they obtained 77% mutations whereas in T1 generations they obtained 70-100% mutations which signified the importance of the CRISPR/Cas9 system for efficient mutant development in barley [30]. These approaches using the CRISPR/Cas9 system might enable advance precision plant breeding and increase crop productivity in cereals which may help to strengthen food security.

CONCLUSION

CRISPR/Cas9 based genome editing system offers many avenues to scientists to modify the sequence of interest in the plant genome. CRISPR/Cas9 genome editing system has been widespread in the plant science field within the past few years and utilized in many studies to improve the cereal crops. Although, off-target effects should be taken into account, modifying the agriculturally important cereal crops would bring the promising green revolution by solving issues like fixing nitrogen, improving nutrition uptake, biofuel productions, and photo-synthetic capability in the near future. Overall, the CRISPR/Cas9 based genome editing system poised to offer several possibilities to improve the cereal crops to overcome the adverse effects of climate change and may help to strengthen the food security in the developing countries.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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