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CRISPR/Cas9 system: Current applications and future potential in rice breeding

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Abstract

Rice (*Oryza sativa* L.) plays a key role in human social and economic life. In order to meet the increasing needs of human food consumption, there is a constant requirement to develop rice cultivars with enhanced agricultural traits. The emerge of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins (CRISPR/Cas9) system provides unprecedented opportunities in studying gene functions and creating new rice varieties with better characteristics, including improved tolerance to biotic and abiotic stresses, and increasing yield and quality. This review aims to provide details about the latest results of CRISPR/Cas9 system application on rice to obtain better adapted to environmental and commercial demands.

SCAN ME

Introduction

Rice (*Oryza sativa* L.) is one of the most important stable food and have contended a pivotal role for human life. Because of its main contribution to the world's energy supply as one of the main daily food for more than half of the human population [1] and its versatile characteristics to multiple environmental conditions [2], rice has been regarded as a strategic crop plant by Food and Agriculture Organization (FAO) [3]. However, rice production is currently facing multiple demands such as climate change, biotic and abiotic stress, and shortage of arable land. As a result, the rice yield is continuously decreasing in recent years. Additionally, rapid human population growth is estimated to expand to 9 billion in 2050, increasing by 34%. Therefore, global rice consumption is projected to rise 45% in the next 30 years, which is equivalent to 450 million tons in 2020 to 650 million ton by 2050 [4,5]. To meet these challenges, enhancing rice resistance to various stress factors and improving rice productivity is a matter of great importance.

In the last few decades, progresses in breeding approaches, especially forward genetic approaches, have played vital roles in elucidating the molecular mechanism that influence agricultural traits of interest in rice. Traditionally, in forward genetic approaches, rice mutations can be mutagenized by chemical (ethyl methane sulfonate-EMS), physical irradiation, (heavy-ion beams or gamma rays) and bacterial genetic insertion (T-DNAs and transposons) [6,7]. However, these methods have limitations. For examples, since EMS and irradiation-generated mutations often lead to mosaicism at the first generation (M_1), it is impossible for forward genetic screening to detect any desirable phenotypes at the M_1 generation. Similarly, because of the heterozygosity in T_0 generation, screening the mutant phenotype caused by T-DNA/transposon has to be conducted at T_1 generation. However, with the advent of RNA interference-based screens, only one generation is needed for the identification of causative mutations for mutant phenotypes. Nevertheless, off-targets effect and instability are the main disadvantages of RNA interference-based mutagenesis [8]

Other restrictions of forward genetic approaches also create obstacles in deciphering important crop traits in rice. For instance, constructing a mapping population is often required to narrow down the causal mutations in an EMS mutants or irradiation mutants. For the untagged T-DNA/transposon mutants, map-based cloning is sometimes involved, while for the tagged T-DNA/transposon mutants, identifying the T-DNA-flanked sequences is sometimes difficult due to the truncation or tandem repeats of the T-DNA fragments [8]. Taken together, since rice plants usually take 3 to 6 months from germination to maturity, establishing the relationship between their phenotypes and the genotypes is often laborious and time-consuming.

The newly developed technologies in genome-editing have provided more tools to address the limitations of traditional breeding methods in elaborating functional genomics and crop improvement in rice. These genetic innovations provide more accurate, time-saving, efficient targeted genomic modifications, including whole-gene insertion or deletion, stacking or pyramiding of genes, in a transgene-free manner [9,10]. The principles of genome-editing approaches are the production of double- stranded breaks (DSBs) of targeted DNA and the introduction of cellular repair of DNA damages [11]. The repair mechanism of DSB is predominantly processed through two pathways, i.e. Non-Homologous End-Joining (NHEJ) and Homology-Directed Repair (HDR) [8]. The NHEJ is the error-prone pathway which mainly causes insertions or deletions, thus results in frameshift mutations, or gene knockouts. On the other hand, the HDR pathway is much more precise in repairing by relying on the exchange of the homologous sequences surrounding the DSBs which contributes to the gene replacement. Regardless of the pathways used, DSBs are initiated by site-specific nucleases, including zinc-finger nuclease (ZFNs), transcription activator-like effector nucleases (TALENs) and the recently discovered clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins (CRISPR/Cas9) [12,13]. Although ZFNs and TALENs approach have been successfully applied in molecular breeding in rice, they also have distinctive limitations in plasmid construction and are expensive.

Methods

Literature search strategy and selection criteria

Google Web, Google scholar, NCBI Databases and OMIC Tools were used to obtain data for this review paper. Different key words were used to retrieve the required research articles and bioinformatics-based information, such as "CRISPR/Cas9'' and "CRISPR/Cas9 in rice''. Research papers consulted for this review were those published over last 10 to 15 years and information regarding CRISPR/Cas9 application in rice was considered for current review.

Discussion

1. CRISPR/Cas9 - MEDIATED GENOME EDITING SYSTEM

The latest CRISPR/Cas9 is extensively employed in genome editing research thanks to its reliability, efficiency and simplicity [14]. Basically, CRISPR/Cas9 is a RNA- mediated adaptive immune system that can be found in bacteria, and archaea [15]. This immune protection provides resistance against genetic attacks and later stores infection histories in a form of spacer sequences for future safety. These spacers function in concert with Cas9 endonuclease proteins to monitor, recognize and degrade exogenous DNA. This process can be divided in three stages: spacer acquisition, biogenesis and immunity. In the spacer acquisition stage, the foreign DNA is identified, captured and embedded into the CRISPR locus in a form of spacer. Subsequently, the expression of the CRISPR/Cas9 system will be initiated in the biogenesis stage, in which the primary CRISPR-RNAs (crRNAs) is synthesized from the CRISPR locus and subsequently undergone many processes to become crRNAs. Finally, in the

immunity stage, the crRNAs, together with the trans-activating RNAs (tracrRNAs), will associate with Cas9 endonuclease, forming a ribonucleotide complex. This complex will initiate interference and consequent degradation of the targeted foreign DNA by base pairing recognition mechanism and endonucleases, respectively.

It was not until the work of Jinek *et al*. (2012), the significant contribution of CRISPR/Cas9 technology to genome editing begins to emerge by the establishment of the programmable version of CRISPR/Cas9 [16]. This modified version of CRISPR/Cas9 is made up of the customizable single strand RNA (sgRNA), which is the fusion product of crRNA and tracrRNA, the recombinant Cas9 protein and. This combination will result in Cas9/sgRNA complex that targets and initiates DSB at specific DNA sequences. Once DSBs are introduced, NHEJ or HDR strategy is activated to repair the DNA damages, leading to gene knockout, or gene knock-in, respectively [8,17]. CRISPR/Cas9 system has been widely employed in various research model research, including Prokaryotes (*Escherichia coli*) [18] and Eukaryotes (*Saccharomyces cerevisiae, Drosophila melanogaster, Caenorhabditis elegans, Arabidopsis thaliana*, etc.) [19-22].

2. CRISPR/Cas9 AND ITS APPLICATION IN RICE IMPROVEMENT

Besides its contribution to human food consumption, rice has been widely employed as excellent model research plant because of its availability of small genome, richness of genetic resources, and genomic synteny with other major crops. As such, there are more than 80 different publications involved in CRISPR/Cas9-mediated genome editing in rice [23]. The results from these papers have shed more lights in understanding the functions of important genes and their potentials. This knowledge has been realized in improving productivity traits, including abiotic stress, herbicide tolerance, disease resistance, yield increase and nutrition enhancement in rice and other agriculture plants.

2.1 Abiotic Stress

CRISPR/Cas9 system has been utilized in some reports to elucidate the functions of genes in response to cold, salinity and drought stresses.

2.1.1 Cold Stress

Annexins (OsANN3) are calcium-dependent phospholipid-binding proteins that participate in plant development, various cellular responses including the negative regulation the cold tolerance. The engineered sgRNA targeted and degraded second exon of OsANN3 gene, which subsequently was repaired by NHEJ strategy [24]. Homozygous mutant lines harboring the induced mutations were tested and presented more susceptibility to cold stress than the wild type, illustrating OsANN2 is the negative regulator of cold tolerance-involved mechanism. Similarly, another transcription factor TIFYb participated in cold stress was mutated in CRISPR/Cas9 experiments performed by Huang *et al*. (2017). Homologous mutant lines indicated the role of TIFYb protein of rice adaptation in low temperature environment [25].

2.1.2 Drought Stress

The phytohormone ABA is known to mediate the responsiveness to many stress processes in plant, including drought. By using CRISPR/Cas9 system to create mutations in third exon of OsSAPK2, the function of OsSAPK2 in the ABA-related drought stress pathway was successfully elaborated. After subjecting the homozygous mutant lines to drought experiments, the results revealed that *sapk2* mutants were ABA-sensitive, and exhibited more sensitivity to drought stress compared to the wild type. Taken together, this reveals OsSAPK2 negatively mediates the drought stress in rice [26].

2.1.3 Salinity Stress

The function of transcription factor OsRAV2 protein in saline stress is well-documented. In one study by Li *et al*. (2020), the authors have stressed on the importance of the promoter region of this gene, GT-1, known for the salt induction. By designing a sgRNA to target this GT-1 element by CRISPR/Cas9 system, after Agrobacterium-mediated transformation, homozygous mutant lines were not able to express OsRAV2 under environment of high salinity. This illustrates the importance of this promoter GT-1 region in saline stress [28].

2.2 Biotic Stress

Disease caused by biotic stress agents, including bacteria, can have harmful influence on rice production. Several genes have been recently mutated by CRISPR/Cas9 system to increase the disease resistance in rice.

2.2.1 Bacterial leaf blight disease

Bacterial leaf blight disease, one of the main threats in rice production, is triggered by the bacteria *Xanthomonas oryzae pv. Oryzae* (Xoo). When entering the host cells, the pathogen will hijack the cellular resources, including a group of type II TAL (transcription activator-alike), to induce the host's gene expression to satisfy the pathogen needs. Some of the targets of these TALs are sugars will eventually be exported transporters (SWEET) genes, which encode sugar transporter proteins. In order to test the role of OsSWEET13 in *Xoo* resistance in rice, sgRNA was engineered to target the first exon of the gene. The mutant lines depicted an increase in *Xoo* resistance, and less severe symptoms compared to the wild type. This concludes OsSWEET is a promising candidate gene in engineering rice cultivars with low susceptibility to bacterial leaf blight disease [34].

2.2.2 Rice Blast

The most detrimental rice disease is rice blast caused by ascomycetes fungus *Magnaporthe oryzae*. Rice blast is documented to damage from 10% to 30% loss in rice production and is found widespread in all rice-growing

Application perspectives	Studied gene	Gene symbol	Transformation method	Reference
Abiotic stress	5-enolpyruvylshikimate-3-phosphate synthase	EPSPS	Agrobaterium-mediated transfer	[27]
	Serine/threonine-protein kinase	SAPK2		[26]
	AP2/ERF domain containing RAV (related to ABI3/VP1)	RAV2		[28]
	Annexin 3	ANN3		[24]
	Bentazon-sensitive-lethal	BEL		[29]
Biotic stress	acetolactate synthase	ALS	Agrobaterium-mediated transfer	[30]
	ethylene response factor 922	ERF922		[31]
	broad-spectrum resistance Kitaake-1	Bsrk1		[32]
	betaine-aldehyde dehydrogenase 2	BADH2		[33]
	Sugars will eventually be exported transporters 13	SWEET13		[34]
	exocyst subunit SEC3A	SEC3A		[35]
	translation initiation factor 4	eIF4G		[36]
Yield and productivity	Glutathione synthetase 3	GS3	Agrobaterium-mediated transfer	[37-39]
	Ideal Plant Architecture 1	IPA1		
	Dense and erect panicle 1	DEP1		
	Grain number 1a	Gn1a		
	Grain width 2 GW2, GW5, TGW6,	GW2 GW5		[32]
	thermosensitive male sterility 5	TMS5		[40]
	Heading date 2, 3, 4	Hd2, Hd4, Hd5		[41]
	Carotenoid cleavage dioxygenase 7	CCD7		[42]
	Lipoxygenase 1, 2, 3	LOX1, 2, 3		[43]
	Carbon starve anther	CSA		[44]
	pyrabactin resistance-like	PYL		[45]
Nutrition	natural resistance-associated macrophage protein 5	NRAMP5		[46]
	Starch branching protein	SBE		[47]
	granule-bound starch synthase	WAXY		[48]
	Omega-6 fatty acid desaturase, endoplasmic reticulum isozyme 1	FAD2-1		[49]
	Nitrate transporter	NRT1.1B		[50]

Table 1: Application of CRISPR/Cas9 system in rice

countries. Mountains of evidence, including know-down RNAi expression, portrayed that ethylene responsive factors (ERFs) negatively regulate *M. oryzae* resistance. Through CRISPR/Cas9 system, one sgRNA was customized to disrupt the first exon of ERF gene. The mutant lines exhibited enhanced resistance to *M. oryzae* compared to the wild type [31].

In another paper, to explore the function of OsSEC3A in rice blast defense, Ma *et al*. (2018) engineered two sgRNAs to aim for the third and tenth exon of the gene. The mutant lines demonstrated declined or impaired agricultural traits, including smaller seedlings, shorter main roots, decrease in plant height, panicle length, tiller number, 1000-grain weight and spikelet fertility. However, significant enhancement of *M. oryzae* resistance was observed. Due to this augmented defense against rice blast, OsSAC3A could be also prominent candidate gene in future research [35].

2.2.3 Rice Tungro Disease

Rice tungro disease is another important rice disease that creates destructive rice production loss in many Asian countries. The interaction of two different viruses, i.e. rice tungro spherical virus (RTSV) and rice tungro bacilliform virus (RTBV), is thought to cause this disease. Traditional breeding approach has illustrated that the translation initiation factor 4 gamma (eIF4G) known to be involved in RTSV resistance. Data from 2018 generated by Macovei *et al*. (2018) indicated that IR64 mutant line has been generated as an attempt to create new source of RTSV resistance. As a result, these novel eIF4G allele-containing mutant lines can be further studied and developed more diverse RTSV-resistant cultivars [36].

2.2.4 Herbicide Tolerance

Herbicide resistance is another important agricultural trait that has been recently studied and introduced into rice. Acetolactate synthase 1 (ALS1) and 5-enolpyrylshikimate-3-phosphate synthase (EPSPS) are the main target of important herbicides such as chlorsulfuron, bispyribac sodium and glyphosate. While ALS1 is an essential enzyme responsible for biosynthesis branched amino acids, EPSPS participates in the bio-production of auromatic amino acids. CRISPR/Cas9 system has been utilized to mutate ASL1 and EPSPS by customizable gRNAs. Phenotypic screening showed that *asl1*, and *epsps* mutant lines were resistant to herbicide and grew normally [27]. Thus, CRISPR/Cas9 system could efficiently generate herbicide resistant rice plants within one generation.

2.3 Yield Improvement

Grain yield is a complex agricultural trait governed by many different genes and environmental factors. Six genes including Gn1a, DEP1, GS3, GS5, GW2, IPA1, and TGW6 are documented to function in grain number, panicle architecture, grain size and plant architecture, respectively. These genes were independently targeted by CRISPR/Cas9 system and the results showed that individual mutant lines harboring their respective edited genes demonstrated increases in grain number, dense erect panicles, and larger grain size [37].

Another line of evidence also reconfirmed the efficiency of CRISPR/Cas system on mutating Gn1a and GS3 [38]. However, only three out of ten novel genotypes lines displayed higher grain number and grain size, suggesting that genetic background could also play a crucial role in masking the expressed mutated genes. Similarly, inducing mutations in three genes GS3, GW2 and Gn1a resulted in higher yields per panicle in two of out three studied varieties, indicating that different

cultivars could affect the expression of the CRISPR-Cas9 mediated target genes [39].

Rice heading date (Hd) is another trait controlled by multiple genetic and environmental factors. Three genes Hd2, Hd4 and Hd5 has been revealed as negative regulators of the heading date of rice. Three different sgRNAs were designed to simultaneously disrupt these genes in a single plasmid. After Agrobacterium transformation, homozygous triple mutant lines showed significantly shortened heading date from 5-30 days compared to the wild type [41].

CRISPR/Cas9 system also can be used to facilitate the development of heterosis in hybrid rice. Hybrid rice is a type of rice that has been bred from two very different parents. It can significantly gain better yield other rice varieties. Hybrid rice contributes from 10% to 20% increase in yield over conventional rice; therefore it has a crucial role in global rice production. Thermo-sensitive 5 (TM5) gene was targeted by CRISPR/Cas9 to develop new thermos-sensitive genic male-sterile (TGMS) lines within one year as maintainer lines under permissive conditions. Similarly, carbon starved anther (CAS) gene, which exhibits male sterility and male fertility, depending on short days or long days conditions, respectively. CAS were also mutated to create reverse photoperiod-sensitive genic male-sterile (PGMS) [40]. Taken together, acceleration of TGMS lines breeding can be achieved by CRISPR/Cas9 system and further developed toward large scale application in two-line hybrid rice breeding.

In rice, yield shares overlapping crucial phytohormones and signaling networks with growth. Previous works have revealed the crucial role of the abscisic acid and its receptors in growth control. In order to provide an alternative genetic strategy to improve rice yields, Miao *et al*. (2018) utilized CRISPR/Cas9 system to target two groups of pyrabactin resistance 1 (PYR1)/PYR1-like (PYL)/regulatory components of (ABA) receptor (RCAR) family proteins, including group I (PYL1-PYL5, and PYL12) and group II (PYL7-PYL11 and PYL13). Although mutants in group II displayed wild-type phenotypes, group I mutants exhibited increased growth and productivity, while maintaining other important traits [45].

2.4 Nutrition Improvement

Rice provides a significant source of daily nutrition for more than half of the world human population. As a result, there is an urgent need to create new rice cultivars enhance the nutrition. Amylose content (AC) and resistant starch (RS) are two of the interest nutrition traits that researchers focus on. AC is arguably the most important quality indicator in rice, and the rice classification based on AC is determined based on the AC. Depend of the consumer demands, the AC and the RS level can be manipulated by the application of CRISPR/Cas9. In the case of reducing AC, AC negative regulator waxy gene were targeted to develop loss of function mutant by CRISPR/Cas9. The homozygous mutant lines *waxy* showed decreased AC while demonstrated no differences in other agricultural traits compared to wild type, including plant height, grain number per panicle, panicle number per plant, yield per plot, grain width, grain length and 1000-grain weight [48].

In another study, CRISPR/Cas9 has been successfully employed to increase AC and RS by mutating two starch branching enzymes (SBEs), including SBEI and SBEII. While sbeI mutants were not distinguishable from the wild type, sbeII showed significantly higher content in AC and RS, indicating SBEII is a key gene in mediating the nutritional aspect of rice regarding AC and RS [47].

Rice brain oil (RBO) is a commercial derivative product from rice that is highly appreciated in Asian countries as cooking oil. One of the main components in RBO is oleic acid, which will be converted to linoleic acid by fatty acid desaturase 2 (FAD2) gene. Of the four FAD2 gene in rice, FAD2-1 is found highly expressed in rice seed. As a result, the knock-out of FAD2-1 gene by CRIPR/Cas9 system is hypothesized to augment the oleic acid content in rice. A work by Abe *et al*. (2018) demonstrated that *fad2-1* mutants exhibited the two-fold increase of oleic acid content compared to the wild type [49].

Beside improving nutrition by enhancing beneficial factors, decreasing toxic components, such as Cadmium (Cd), is also another aim in rice breeding. Since Cd is highly toxic heavy metal element that poses a serious threat to human consumption, its excessive presence and accumulation in rice need to be resolved. One of the strategy is to mutate the metal transporter gene NRAMP5 using CRISPR/Cas9 system. NRAMP5 regulates the root uptake of Cd, thus knock-outing this gene can reduce the Cd content. Two sgRNAs were engineered to target the ninth exon of NRAMP5. Homozygous nramp5 mutants displayed less Cd quantity and retained wild-type-like traits when grown in Cd-contaminated fields, indicating NRMAP5 is a good candidate gene in development rice cultivars with low Cd content [46].

CONCLUSION

CRISPR/Cas9 system has emerged as the most effective tool for crop improvement thanks to its accuracy in creating mutations at genes of interest. By generating a genome-wide mutant library, functional characterization of more than 12000 unknown genes can be achieved. Therefore, incorporating beneficial genes in developing elite cultivars for the breeding process not only in rice, but also in other important crops, can be significantly accelerated. Another major advantage of this process is the precision in eliminating the transgene at the first generation through genetic segregation. Consequently, the transgene-free mutant plants, for instance waxy corn, flavored tomato, can be exempted from the biosafety regulation rules by the USDA.

However, there are still some challenges of CRISPR/Cas9 application in rice that researchers need to mitigate. The first challenge is the requirement of tissue culture in introduction of foreign DNA. This can pose difficulties in some commercial varieties, especially *indica*. Moreover, the complications during tissue culture including labor-insensitivity, tedium, can create

somaclonal variations which can compromise the overall of regenerated plants [9]. Second challenge is the disadvantages of the delivery system for CRISPR/Cas9, i.e. *Agrobacterium* and particle bombardment- mediated transformation. While Agrobacterium-mediate gene transformation is only efficient to limited genotypes within species, particle bombardment- mediated transfer can be productive in a broader range of genotypes. Nevertheless, plant regeneration following bombardment is restricted [9]. The third challenge is the occurrence of undesired off-target events. Although the precision of targeting genes of interest has been enhanced by the introduction of higher specificity endonuclease than Cas9, such as Cpf1 [9,14], gene replacement editing is still difficult because of the low efficiency of HR in plants. The fourth challenge is the field adaptation of the CRISPR/Cas9-mediated genetic modified plants. A majority of the CRISPR/Cas9- related works in rice are only conducted in confined lab conditions. There can be complications once these edited plants are tested on field trials [14]. Therefore, more studies of edited plants on the field can help answer this challenge.

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Conflict of interest

The author declare that there is no competing interests.

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