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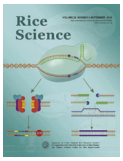

Review

CRISPR/Cas9: Development and Application in Rice Breeding

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Abstract: Rice (*Oryza sativa* L.) is an important staple food crop worldwide due to its adaptability to different environmental conditions. Because of its great economic and social importance, there is a constant requirement for new varieties with improved agronomic characteristics, such as tolerance to different biotic (such as bacterium, fungus, insect and virus) and abiotic stresses (such as salinity, drought and temperature), higher yield and better organoleptic and nutritional value. Among the new genome editing technologies, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) (CRISPR/Cas) system allows precise and specific edition in a targeted genome region. It is one of the most frequently used techniques for the study of the function of new genes and for the development of mutant lines with enhanced tolerance to biotic and abiotic stresses, herbicide resistance or improved yield. The wide varieties of applications for this technology include simple non-homologous end joining, homologous recombination, gene replacement, and base editing. In this review, we analyzed how some of these applications have been used in rice cultivars to obtain rice varieties better adapted to current environmental conditions and market requirements.

Key words: rice; CRISPR; Cas; genome editing; biotic stress; abiotic stress; yield; off-target; mutation

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world. The major area of production is Asia, with more than 6.6 billion tonnes produced in 2016, followed by America (36 million tonnes) and Africa (32 million tonnes) (FAOSTAT, http://www.fao.org/faostat/en/#home). One of the advantages of rice is that it can be grown in a wide range of environments, even in areas not suitable for other crops (Breviario and Genga, 2013). However, because of climate change, biotic stress and stressful environmental conditions such as drought, salinity and extreme temperatures have increased all over the world, affecting rice production and causing enormous losses. In addition, the world's population is projected to reach 9.1 billion by the year 2050, increasing by 34%. According to the Food and Agriculture Organization of the United Nations (FAO), the global demand for agricultural products will increase by about 70% by 2050. Annual cereal production need to increase from 2.1 billion today to about 3.0 billion tonnes, in order to feed the projected world population.

Based on these, the main concern in current agricultural research is to improve plant tolerance to biotic and abiotic stresses and increase productivity (Delorge et al, 2014). In recent years, new precise genome editing techniques have been developed and replaced previous techniques such as random mutagenesis, naturally occurring mutations and classical breeding techniques because these methodologies are time-consuming and take too long to obtain individuals with the desired phenotype. New techniques have become important tools for plant science and plant molecular breeding (Endo et al, 2018). Genome editing allows the introduction of deletions, insertions or base substitutions by causing damage, double-

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strand breaks (DSBs), in targeted deoxyribonucleic acid (DNA) (Fig. 1-A). In order to repair this damage, plant cells follow two strategies. The preferred repair pathway in higher plants is non-homologous end-joining (NHEJ) (Fig. 1-B), which mainly causes insertions or deletions (InDels) and can result in frameshift mutations. A second repair pathway, homologous recombination (HR) (Fig. 1-C), occurs when a template with homologous sequence surrounding the DSB is available and is used for DSB repair, resulting in gene replacement (Endo et al, 2018). Regardless of the repair mechanism used, DSBs are induced by sequence-specific nucleases (SSNs), including zinc finger nucleases (ZFNs) (Pabo et al, 2001) and transcription activator-like effector nucleases (TALENs) (Moscou and Bogdanove, 2009). Both SSNs are artificial bipartite enzymes that consist of a DNA-binding domain and the *FokI* nuclease domain. In order to target the nuclease to the desired DNA sequence, a modular DNA-binding domain must be designed and assembled. Although the design and construction of large modular proteins are both laborious and expensive (Belhaj et al, 2015), they have been used for molecular breeding with relative success (Sprink et al, 2015).

The newest and most widely used genome editing technique is based on clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) (CRISPR/Cas). This technique is different from ZFN and TALEN in terms of the DNA-binding system. Cas9 can be targeted to a specific genomic sequence by an easily engineered 20 base pair (bp) RNA guide sequence that binds to its DNA target by base-pairing. The use of different genome editing techniques in rice from 2013 until early 2018 was recently reviewed (Mishra et al, 2018). However, in this review, we will focus on the use of CRISPR/Cas technology to improve desirable traits in rice, such as tolerance to biotic and abiotic stresses as well as different parameters involved in determining yield (Table 1). In addition, we will discuss the pre-requisites, limitations and advantages of this technique in rice.

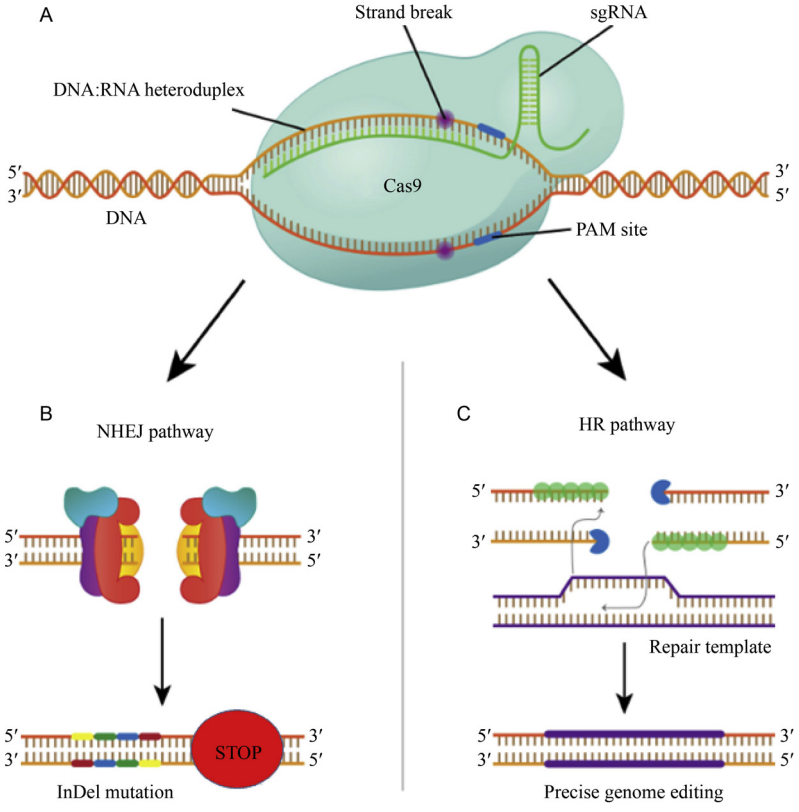

Fig. 1. CRISPR/Cas9 system components and pathways.

A, Components required for genome editing using the CRISPR/Cas9 system: a DNA endonuclease (the most commonly used is the Cas9 protein from *Streptococcus pyogenes*) and a customizable single guide RNA (sgRNA). B, Non-homologous end-joining (NHEJ) repair pathway. C, Homologous recombination (HR) repair pathway.

PAM, Protospacer adjacent motif.

Table 1. Applications of CRISPR in rice from 2013 to mid-2018.

Application	Targeted gene	Strategy	Transformation method/Explant	Reference
Response to bacterial blight	*SWEET14*, *SWEET11*	NHEJ	PEG/Protoplast	Jiang et al, 2013
	SWEET13	NHEJ	*Agrobacterium*/embriogenic calli	Zhou et al, 2015
Rice blast resistance	*EFR922*	NHEJ	*Agrobacterium*/embriogenic calli	Wang et al, 2016
	SEC3A	NHEJ	*Agrobacterium*/embriogenic calli	Ma et al, 2018
Response to saline stress	*RAV2*	NHEJ	*Agrobacterium*/embriogenic calli	Duan et al, 2016
Response to drought stress	*SAPK2*	NHEJ	*Agrobacterium*/embriogenic calli	Lou et al, 2017
Response to cold stress	*ANN3*	NHEJ	*Agrobacterium*/embriogenic calli	Shen et al, 2017
Herbicide resistance	*EPSPS*	Exon replacement by NHEJ	Biobalistic/embriogenic calli	Li et al, 2016a
	AAC	Base editing by nCas9	*Agrobacterium*/embriogenic calli	Li et al, 2018
	ALS	Base editing by nCas9 and/or dCas9	*Agrobacterium*/embriogenic calli	Shimatani et al, 2017
	ALS	HR	Biobalistic/embriogenic calli	Sun et al, 2016
Yield improvement	*Gn1a*, *DEP1*, *GS3*, *IPA1*	NHEJ	*Agrobacterium*/embriogenic calli	Li et al, 2016b
	GS3, *Gn1a*	NHEJ	*Agrobacterium*/embriogenic calli	Shen et al, 2016
	DEP1	Gen deletion by NHEJ	*Agrobacterium*/embriogenic calli	Wang et al, 2017
	GW2, *GW5*, *TGW6*	NHEJ	*Agrobacterium*/embriogenic calli	Xu et al, 2016
	GS3, *GW2*, *Gn1a*	NHEJ	*Agrobacterium*/embriogenic calli	Zhou et al, 2018
	TMS5	NHEJ	*Agrobacterium*/embriogenic calli	Zhou et al, 2016
Fatty acid metabolism	*FAD2-1*	NHEJ	*Agrobacterium*/embriogenic calli	Abe et al, 2018
Flowering time	*Hd2*, *Hd4*, *Hd5*	NHEJ	*Agrobacterium*/embriogenic calli	Li et al, 2017
Nitrogen efficiency use	*NRT1.1B*	Allele replacement by HR	Biobalistic/embriogenic calli	Li et al, 2018
Cadmium accumulation	*Nramp5*	NHEJ	*Agrobacterium*/embriogenic calli	Tang et al, 2017
Starch metabolism	*SBEI*, *SBEIIb*	NHEJ	*Agrobacterium*/embriogenic calli	Sun et al, 2017
	WAXY	NHEJ	*Agrobacterium*/embriogenic calli	Zhang et al, 2018
Transgene excision	*GUS*	NHEJ	*Agrobacterium* and biobalistic/embriogenic calli	Srivastava et al, 2017
Comparison between Cas9 and Cpf1	*EPFL9*	NHEJ	*Agrobacterium*/immature embryos	Yin et al, 2017
Genome wide mutant	12 802 genes	NHEJ	*Agrobacterium*/embriogenic calli	Meng et al, 2017
Library	34 234 genes	NHEJ	*Agrobacterium*/embriogenic calli	Lu et al, 2017

NHEJ, Non-homologous end-joining repair pathway; HR, Homologous recombination repair pathway; PEG, Polyethylene glycol.

CRISPR/Cas system

CRISPR/Cas is a type of adaptive immune system, which degrades exogenous DNA, discovered by accident in 1987 in *Escherichia coli* (Ishino et al, 1987). Later it was reported in other bacteria including *Shigella dysenteriae*, *Salmonella enterica* and *Mycobacterium tuberculosis* (Nakata et al, 1989; Hermans et al, 1991). It was firstly reported in archaea by Mojica et al (1993). But it was not popular until 2012 when the potential to exploit this system for genome editing was suggested (Jinek et al, 2012).

The immune memory of this system is stored in the form of spacer sequences from foreign genomes inserted into CRISPR arrays (Koonin et al, 2017). These spacers, along with Cas proteins, act as a surveillance system to recognize and degrade foreign nucleic acids. This process has three stages. The first stage, called adaptation, immunization or spacer acquisition, consists of the recognition and integration of foreign DNA (spacers) into the CRISPR locus. The sequence of viral or plasmid DNA in the spacer is called the protospacer. Usually there is a short conserved sequence in the immediate vicinity of the protospacer, referred to as the protospacer adjacent motif (PAM). The second stage consists of the expression of the system. A primary transcript (pre-crRNA) is transcribed from the CRISPR locus and processed into small CRISPR RNAs (crRNA). In the final stage, called interference or immunity, crRNAs, along with trans-activating crRNA (tracrRNA), form a ribonucleoprotein complex with Cas proteins. This complex recognizes foreign DNA by base pairing and then degrades it (Bhaya et al, 2011).

CRISPR/Cas system has been engineered and used for genome editing in wide varieties of organisms such as mammalian cells, bacteria, fungi and plants (Jiang W Y et al, 2013; Krappmann, 2017). Two components are required for genome editing using this system: a DNA endonuclease (the most commonly used is the Cas9 protein from *Streptococcus pyogenes*) and a customizable single guide RNA (sgRNA) (Fig. 1-A). Cas9 has a bi-lobed architecture, with a large globular recognition lobe (REC) and a small nuclease lobe (NUC) with two nuclease domains, RuvC and

HNH, which each cut a specific DNA strand. The sgRNA is a small non-coding RNA that is the fusion of crRNA and tracrRNA (Jinek et al, 2012). CRISPR/Cas system can potentially be used to edit any sequence in the genome of any organism. The only requisite is that the target sequence must be adjacent to a PAM sequence. Cas9 from *S. pyogenes* recognizes the sequence 5′-NGG-3′ as PAM. However, there are other Cas proteins with different PAM sequences, such as Cpf1 (or Cas12) that recognizes the sequence 5′-TTTN-3′ or 5′-TTN-3′ as PAM. Moreover, new Cas9 variants (VQR, EQR and VRER) have been developed to recognize alternative PAMs, thereby increasing the possibilities for modifying any target sequence in a genome (Anders et al, 2016). Use of CRISPR/Cas9 system in plants (rice and wheat) was firstly reported by Shan et al (2013). Since then, there have been several reports of successful plant genome editing using this system. One important feature of the system is the ability to act in-trans on the respective target, allowing the separation of the mutagenesis agent (CRISPR protein/gene and sgRNA) from the sequence with the desired modification (Ricroch et al, 2017). In order to do this, it is necessary to obtain stable production of CRISPR/Cas-mutated lines without CRISPR/Cas expression cassettes in the final plants. This can be achieved by several methods such as genetic segregation, transient expression of the CRISPR components, or transformation by ribonucleoprotein complexes (Pyott et al, 2016; Zhang et al, 2016; Liang et al, 2017). The easiest way to achieve this in rice is the genetic segregation of transgene in the next generation, which is the most common method to obtain transgene-free plants. However, transformation with ribonucleoprotein complexes has been also achieved in rice (Woo et al, 2015).

Screening of CRISPR/Cas-generated mutant plants

The increasing interest in this technology has led to the development and/or adaptation of new techniques for screening CRISPR/Cas-generated mutant plants. Chen et al (2018) developed a rapid, cost-effective, high-throughput mutant screening protocol based on Illumina sequencing followed by high-resolution melting (HRM) analysis. First, pooled DNA samples are amplified using primers targeting the sgRNA region, and the PCR products are sequenced. Since nucleotide variant frequency (NVF) is a measure of the frequency of abnormal nucleotides relative to the wild type, samples with high NVF values are considered likely to contain mutants. In this way, it is possible to detect one mutant plant in a 42-plant pooled sample. HRM analysis was then used to identify individual mutant line within the pooled samples. Liu et al (2019) developed a high throughput method to genotype mutant rice plants, based on protocols described earlier (Bell et al, 2014). Briefly, DNA samples are arranged in 96-well microplates, and two PCR steps are performed: the first one with specific primers designed to amplify the expected mutation region, and the second one with general primers coupled to barcodes in order to identify each sample. These PCR products are pooled together and sequenced by any next generation technique. Thus, the sequences generated are analyzed by the pipeline high-throughput tracking of mutation (Hi-Tom), which allows the extraction of primers and barcodes, filters low quality sequences, compares the sequences with the reference genome (wild type) and reports any mutation detected in each sample. Along the same lines, Peng et al (2018) developed a simple high-throughput qPCR-based method that can distinguish wild types from mutant samples and allow the identification of heterozygous and homozygous mutations in several plant species, including *Oryza sativa*, *Arabidopsis thaliana*, *Sorghum bicolor* and *Zea mays*. This technique uses two differently labelled probes for the detection of the same PCR product with one probe located outside the mutation position and the other within the mutation position. In this way, any mutation at the target site will block the binding of the second probe. Using qPCR, wild type, heterozygous and homozygous samples are easily differentiated.

In rice, two new techniques for the identification of CRISPR/Cas-induced mutants have been reported. One uses non-invasive near-infrared (NIR) hyperspectral imaging. Radiation from light sources in the NIR region provides a unique spectral signature of organic molecules. Because modifications in DNA affect this unique spectral signature, wild type and mutant seeds can be differentiated (Feng et al, 2017). The other new technique combines CRISPR/Cas with the RNA interference (RNAi) technique, and takes advantage of the natural resistance of rice plants to the herbicide benzaton (Lu H P et al, 2017). In this technique, an RNAi expression element targeting the gene responsible for benzaton resistance is incorporated

into a CRISPR/Cas9 vector. After *Agrobacterium*-mediated transformation, tillers of T_0 plants are separated and grown as two subplants, one of which is sprayed with benzaton. Then, genome editing is analyzed in tolerant and susceptible plants. Plants carrying the RNAi expression element along with the CRISPR/Cas9 cassette show the mutated genotype (sensitivity to the herbicide). Thus, bentazon susceptibility is 100% correlated with the targeted mutations in T_0 plants (Lu H P et al, 2017).

Pioneer reports of rice genome editing by CRISPR/Cas

CRISPR/Cas technology for genome editing of rice and other plants (*Arabidopsis*, tobacco, sorghum and wheat) was first reported in 2013 (Jiang W Z et al, 2013; Miao et al, 2013; Xie and Yang, 2013). Shan et al (2013) reported that two sgRNAs targeting the rice phytoene desaturase gene *OsPDS* are designed and an efficiency of mutagenesis (15%) is obtained in transformed rice (Nipponbare) protoplasts. Nipponbare calli is bombarded with Cas9 plasmid and sgRNA expression plasmids designed to cleave either *OsPDS* or *BETAINE ALDEHYDE DEHYDROGENASE 2* (*OsBADH2*). Mutations in *OsPDS* are identified in 9 out of 96 independent transgenic plants (9.4%), and mutations in *OsBADH2* are detected in 7 out of 98 transgenic plants (7.1%). HR repair system was evaluated using a single-stranded oligo introduced into *OsPDS* during protoplast transformation, and 2 out of 29 colonies incorporate the designed oligo in the targeted site. Miao et al (2013) used the reporter gene *DGU.US* which restores β-glucuronidase (GUS) activity through single strand annealing upon DNA cleavage between the repeat regions in the *DGU.US* reporter. A sgRNA and a dual-crRNA:tracrRNA targeting this region are designed. Restoration of GUS activity was evaluated in rice calli from the Nipponbare. Both the sgRNA and dual-crRNA:tracrRNA are effective in this assay. Furthermore, the CRISPR/Cas technology was used to disrupt endogenous genes that allow easy identification of mutant plants. The *CHLOROPHYLL A OXYGENASE 1* (*CAO1*) mutant *cao1*, which shows a pale green leaf phenotype, and the *LAZY1* gene mutant were chosen because both mutants exhibit a pronounced tiller-spreading phenotype after the tillering stage. In this analysis, a high mutation efficiency ranging from 83% to 91% is observed in transformed lines of rice variety Kitaake (Miao et al, 2013). Other works demonstrated the ability to edit the rice genome using CRISPR/Cas technology by targeting genes involved in defence responses. Jiang W Z et al (2013) designed sgRNAs to edit the genes *OsSWEET14* and *OsSWEET11*, which are involved in resistance to bacterial blight caused by *Xanthomonas oryzae*. After protoplast transformation, edition of the target sites was confirmed by PCR and sequencing. Xie and Yang (2013) used three sgRNAs targeting the rice *MITOGEN-ACTIVATED PROTEIN KINASE 5* (*MPK5*) gene, a negative regulator of the plant defence response. Protoplasts from the rice variety Nipponbare are transformed and efficiencies ranging from 2% to 10% for different sgRNAs are observed.

Since the publications of these reports validate the use of CRISPR/Cas genome editing in rice, there have been many studies implementing it for different purposes (Table 1).

Pre-requisites and limitations of CRISPR/Cas9 technology for rice improvement

Genome editing in plants requires the introduction of the editing system inside the plant cells. In recent years, there has been a remarkable breakthrough in genetic transformation, and the introduction of foreign DNA is now possible for most major crops, including rice (Jung and Seo, 2017). However, since these techniques involve tissue culture, which is difficult in some rice varieties, especially *indica*, the application of genome editing could be limited in important commercial varieties. Moreover, the development and optimization of tissue culture protocols are labor-intensive and time-consuming procedures. Tissue culture can lead to somaclonal variations that can compromise the overall of regenerated plants (Kaeppler et al, 2000; Sarmast, 2016). Minimizing or eliminating the tissue culture requirement would improve the efficiency of crop transformation and genome editing (Jung and Seo, 2017).

Delivery of CRISPR/Cas elements (Cas9 and sgRNAs) is another step during genome editing in plants. In order to express CRISPR/Cas elements and select genome-edited plants, vectors containing all of the elements and a selectable marker are delivered into the cells. These genes are usually integrated into the cell genome to allow the expression of Cas9 and the sgRNAs. Once the genome-edited plants are identified, these elements can be removed by segregation in the

next generation or by site-specific recombination. An alternative is the transient expression of these elements without incorporation into the host genome. Transient expression could be sufficient for genome editing by CRISPR/Cas elements (Hamada et al, 2018). Another alternative is the use of ribonucleoprotein complexes instead of the expression cassette of the elements. In this way, transgene-free mutant plants can be obtained from the beginning. This strategy has been tested successfully in some crops including rice (Woo et al, 2015; Svitashev et al, 2016; Liang et al, 2017, 2018). Regarding delivery systems, *Agrobacterium*- and particle bombardment-mediated transformation are the most widely utilized. However, both systems have their pros and cons. Efficient *Agrobacterium*-mediated transformation is limited to a narrow range of genotypes within a species. Particle bombardment-mediated gene transfer can be applied to a wider range of genotypes than *Agrobacterium*-mediated gene transfer, but plant regeneration after bombardment can be limiting (Altpeter et al, 2016). Therefore, the improvement of these techniques or the development of new technical advances can help in the development of genome-edited lines.

The main concern about genome editing using CRISPR/Cas technology is the occurrence of undesired off-target events. This has been an important barrier to the application of genome editing systems in plant breeding. The use of new nucleases with higher specificity than Cas9, such as Cpf1, has improved this aspect of genome editing. Yin et al (2017) compared the two nucleases for genome editing in the *indica* variety IR64 using CRISPR/Cas9 and CRISPR/Cpf1 and indicated that both systems are equally efficient for editing the *EPIDERMAL PATTERNING FACTOR LIKE 9* (*EPFL9*) gene. T_0 plants generated by *Agrobacterium*-mediated transformation of immature embryos show mutation frequencies (heterozygous) of 4% and 10% for Cas9 and Cpf1, respectively. However, T_1 plants show a higher number of homozygous mutant plants with Cas9 (25%–28%) than with Cpf1 (16%–20%). Off-target effects are analyzed in T_2 homozygous transgene-free plants using the Cas9 system and no off-target effects are observed (Yin et al, 2017).

Recently, whole genome sequences of 34 edited rice (Nipponbare) plants using Cas9 (12 sgRNAs) and 15 edited plants using Cpf1 (3 crRNAs) were analyzed for off-targets. Both T_0 and T_1 plants are analyzed and only one Cas9 sgRNA (about 8%) results in off-target mutations in T_0 lines at predicted sites. None of the T_1 plants shows off-target mutations with Cas9 or Cpf1. Moreover, several mutations due to the tissue culture process are observed (approximately 102–148 single nucleotide variations and 32–83 inserts/deletions per plant). These results indicate that both enzymes are very specific in generating target mutations (Tang et al, 2018).

Advantages of CRISPR/Cas9 system

One of the most appealing characteristics of CRISPR/Cas system is its multiplex editing capability, which leads to the mutation of several genes in the same transformation event, thereby reducing the time and cost of generating new varieties. This trait has been applied for the simultaneous mutation of an entire gene family in rice (Nipponbare and Kitaake). Eight sgRNAs targeting four members of the mitogen-activated protein kinase gene family have been designed. Each pair targets two genomic sites within a gene locus, 350–750 bp apart, so that excision could be observed in edited cells. Numerous sgRNAs are produced from a single polycistronic gene using the endogenous tRNA-processing system, which precisely cleaves both ends of the tRNA precursor. After protoplast transformation, analysis of the target genes reveals efficiently excised chromosomal fragments at all the four targeted loci with 4%–20% frequency. When the same construct is used to transform embryogenic calli by *Agrobacterium*, transformed plants show mutations at all the eight sites. However, fragment deletion is detected only at two loci (Xie et al, 2015). With this system, simple, double and quadruple mutants are obtained. Mutation efficiency is 86%–100% for simple mutants, 67%–100% for double mutants and 86% for quadruple mutants. Phenotypic analysis of the mutants reveals severely dwarfed and sterile *mpk1* mutants and defective embryo development in homozygous *mpk1* seeds from heterozygous parents. In contrast, heterozygous *mpk6* mutant plants completely fail to produce homozygous *mpk6* seeds (Minkenberg et al, 2017).

Another important advantage of CRISPR/Cas is that transgene-free mutant plants can be obtained in the first generation. This can be achieved by segregation of the transgene. In rice, there are several examples of T_1 transgene-free mutant plants (Li J et al, 2016; Wang et al, 2016). Another way to obtain transgene-free mutant plants is by transformation with ribonucleoprotein complexes (Cas9 and sgRNAs) for DNA-free genome

editing. In this way, rice (Dongjin) protoplasts are transformed with Cas9 coupled to a sgRNA targeting the cytochrome *P450* gene, and a mutation frequency of about 20% is achieved (Woo et al, 2015).

Recently, a new rice transformation system was developed in order to obtain T_1 transgene-free plants. This system takes advantage of a pair of suicide transgenes that effectively kills all CRISPR/Cas-containing pollen and embryos produced by T_0 plants. One of these genes is the bacterial *BARNASE* gene, which encodes a toxic protein that kills plant cells. This gene is under the control of the *REG2* promoter, which is expressed during early embryo development. This system contains the gene *CMS2*, encoding a rice male gametophyte specific lethal protein under the *CaMV 35S* promoter. In this way, T_0 plants containing these expression cassettes along with the CRISPR/Cas9 system will produce toxic proteins that kill male gametophytes and embryos, and only the transgene-free seeds will be produced. This construct is named Transgene Killer CRISPR (TKC) (He et al, 2018). In order to test this system, a sgRNA is designed to target the gene *LAZY1* because *lazy1* mutants show a large tiller angle and allow an easy way to identify mutant individuals. After *Agrobacterium* transformation of rice calli, 65 T_0 plants are obtained and 29 show *lazy1* phenotype. Seeds from 10 T_0 plants are evaluated for transgenes and none of them show the presence of TKC or CRISPR/Cas systems. The mutation segregation pattern does not correspond to the expected Mendelian ratio. However, every single T_1 plant contains mutations at the target site and these plants were either homozygous or bi-allelic, demonstrating the 100% efficiency in eliminating both the LAZY1 function and the transgenes (He et al, 2018).

Applications of CRISPR/Cas in rice

A highly interesting application of this technology is the generation of a genome-wide mutant library that can be used for identifying gene functions and for genetic improvement. Meng et al (2017) searched the rice genome for every potential sgRNA, taking into consideration that the seed region (12 bp adjacent to PAM) should match only once in the genome in order to avoid off-targets. They found 1 535 852 target sites located in the exon regions of 52 916 rice genes. In order to enrich loss-of-function mutations, sgRNAs located in exons near the beginning of ORFs are selected and two sgRNAs in each candidate gene are chosen. In this way, 12 802 genes and 25 604 sgRNAs are generated. The plasmid library is checked for quality, gene coverage and sgRNA accuracy by sequencing. Then, it is transformed into *Agrobacterium* for rice (Zhonghua 11) transformation and more than 14 000 independent T_0 lines are obtained. A total of 182 T_0 plants are randomly chosen and sequenced to evaluate the quality and coverage of the mutants. Of these, 139 plants harbouring single correct sgRNAs are found, of which 136 are different, indicating a good representation of every sgRNA. Finally, predicted off-targets were checked for some sgRNAs and no off-target mutations are found (Meng et al, 2017). In a similar way, Lu Y M et al (2017) designed a sgRNA library with 88 541 members, targeting 34 234 genes. There is an average of 2.59 sgRNA per gene in the rice cultivar MSU7. The library construction is checked by sequencing. Thus, more than 90% of the plasmid is confirmed and the coverage of 99% is observed. It is transformed into *Agrobacterium* for rice (MSU7) transformation. A total of 84 384 transgenic plants are obtained with a mutation frequency of 83.9%. A high-throughput genotyping is performed as described by Bell et al (2014). In this way, 9 216 plants are analyzed and 7 004 are successfully identified (PCR positive), from which 86.5% contains a sgRNA. After all, 5 541 plants are identified and 2 326 loci are covered (Lu Y M et al, 2017). These results showed that the CRISPR/Cas9 system is a powerful tool for constructing mutant libraries in rice and can significantly accelerate the breeding process for the first time. Moreover, it will help in the functional characterization of unknown genes, not only in rice, but in other crops in general.

Another interesting use of CRISPR/Cas is the precise elimination of marker genes in transgenic plants. Because of biosafety concerns, this is especially important when the selection marker gene encodes antibiotic and/or herbicide resistance. Moreover, the presence of selection markers makes the introduction of new genes into transgenic lines more difficult. Therefore, removing and recycling selection markers are highly desirable. For this purpose, two sgRNAs are designed targeting both ends of the *GUS* gene in the B_1 transgenic rice (Nipponbare) line (Nandy and Srivastava, 2012). With this strategy, 5 out of 113 transgenic events show the expected excision of the full gene. Two of these are biallelic excisions. Sequence analysis of these events indicated that the

excision is extremely precise since no mutations are observed in the two cutting sites (Srivastava et al, 2017). This new application of CRISPR/Cas could be used for selectable marker gene excision in rice or other plant species to improve breeding techniques.

Genome editing to improve resistance to biotic and abiotic stresses

Global climate change has increased the areas where adverse biotic and abiotic conditions seriously threat rice production and cause huge loss around the world. The International Food Policy Research Institute (IFPRI) forecasts that by 2050 rice yield loss could be about 13% worldwide, with the most affected areas being middle east-north Africa, Latin America and the Caribbean. The loss will cause rice price increased by 32% to 37%. These adverse conditions include stresses caused by drought, salinity, extreme temperature, flooding, low nutrient availability, virus, bacterium, fungus, nematode, insect and herbivore. Drought and salinity are the main abiotic causes of rice yield loss worldwide. Blast disease, caused by *Magnaporthe oryzae*, and bacterial blight, caused by *Xanthomonas oryzae*, are the most destructive rice diseases.

Tolerance to biotic stress

Resistance to *X. oryzae*

X. oryzae utilizes a group of type III TAL (transcription activator-like) effectors to induce host gene expression and condition host susceptibility. Some of the targets of these effectors are SWEET genes, which have been shown to be sugar transporters. Zhou et al (2015) tested the role of the *OsSWEET13* gene in rice resistance to *X. oryzae*. A sgRNA is designed to target the first exon of the gene. After *Agrobacterium*-mediated transformation of rice calli (Kitaake), two mutant lines with deletions of 4 and 11 bases in the coding region of *OsSWEETB* are selected. These lines show the same phenotypic characteristics as control plants in normal growth conditions. However, they show increased resistance to *X. oryzae* and lesion length is reduced by about 90% compared to wild type plants in disease occurrence (Zhou et al, 2015).

Resistance to *M. oryzae*

Rice blast is the most devastating disease in all rice-growing countries, with typical loss of 10%–30%, although regional epidemics can be devastating (Dean et al, 2012). Therefore, augmenting resistance to *M. oryzae* is one of the most effective approaches for managing this disease. There is evidence that plant ethylene responsive factors (ERF) are involved in the modulation of multiple stress tolerance (Müller and Munné-Bosch, 2015). Moreover, knockdown expression of rice *ERF922* from accession Zhonghua 17 by RNAi enhances resistance to *M. oryzae*, indicating that this gene may act as the negative regulator of resistance (Liu et al, 2012). CRISPR/Cas9 is used to mutate the *OsERF922* gene. Through *Agrobacterium* transformation of rice calli (Kuiku 131) using one sgRNA targeting the first exon of the gene, 21 mutant lines are obtained with different types of mutations, including deletions (64.3%), insertions (23.8%) and both (11.9%). Due to segregation, mutant homozygous transgene-free rice plants are obtained in the T_1 generation. The analysis of different agronomic traits (plant height, flag leaf length and width, number of productive panicles, panicle length, number of grains per panicle, seed-setting rate and 1000-grain weight) of these mutants shows no differences with the wild type. However, mutant lines show enhanced resistance to *M. oryzae* compared to the wild type. Lesion length in the mutant lines is about 66% smaller than that in the wild type (Wang et al, 2016). The possibility of using several sgRNAs targeting different regions of the same gene is also analyzed. Thus, using three sgRNAs, 90% of the plants carry mutations in the three different sites at the same time, which suggests that this system allows highly efficient multilocus mutation in rice plants.

In a different line of evidence, Ma et al (2018) explored the role of the *OsSEC3A* gene in the defense response to *M. oryzae*. *OsSEC3A* is an important subunit of the exocyst complex in rice. In this case, the authors designed two sgRNAs targeting the third and tenth exons of this gene. Five rice (Kitaake) mutant lines are obtained showing deletions, insertions or both in the targeted sites. These lines show altered growth and agronomic traits compared to the wild type, with smaller seedlings, shorter main roots, and decreased or impaired agricultural traits (plant height, panicle length, tiller number, 1000-grain weight and spikelet fertility). One line is chosen for further analysis; however, no transgene-free plants are obtained. This mutant line shows enhanced activation of the defence response, as evidenced by up-regulation of pathogenesis-related proteins and salicylic acid synthesis-related genes and increased levels of salicylic acid. Due to this augmented defence state,

ossec3a mutants are more tolerant to *M. oryzae* infection (Ma et al, 2018).

Tolerance to abiotic stress

There are some reports about the use of CRISPR/Cas to study the role of genes involved in the rice response to different abiotic stresses.

Salinity tolerance

Duan et al (2016) studied the promoter of the *OsRAV2* gene, a transcription factor involved in the response to saline stress. A specific region of this promoter, *GT-1*, which is essential for the salt induction of this gene, is identified. In order to elucidate the role of this element, a sgRNA is designed to target the *GT-1* region of the promoter. Two out of twelve rice (Nipponbare) lines are selected because they have lost the *GT-1* element. The inability of the mutant lines to overexpress the *OsRAV2* gene under conditions of high salinity confirms the importance of this region.

Drought tolerance

Lou et al (2017) elucidated the role of *OsSAPK2* (osmotic stress/ABA-activated protein kinase 2) using loss-of-function mutants by CRISPR/Cas. A sgRNA is designed to target the third exon of the gene. After *Agrobacterium*-mediated transformation, 20 transgenic T_0 lines are obtained. Two homozygous T_1 mutant plants are obtained from these lines and characterised by sequencing the target region. The *sapk2* mutants exhibit an ABA-insensitive phenotype and are more sensitive to drought stress than the wild type, indicating that *OsSAPK2* is important for the response to drought conditions in rice.

Cold tolerance

The role of the rice annexin *OsANN3* during cold stress was studied by Shen et al (2017). In this case, the second exon of the gene is targeted by the sgRNA. After *Agrobacterium*-mediated transformation, the target regions are sequenced in 19 transgenic T_0 rice (Taipei 309) lines to confirm mutations. In this way, six mutants are detected with four types of NHEJ mutations, 1-bp insertion, 1-bp deletion, 3-bp deletion and 4-bp deletion. Of these, 3 lines are homozygous mutants and 1 line is a biallelic mutant. Three T_1 lines from the T_0 homozygous and biallelic mutants are evaluated for cold tolerance, and all the mutant lines tested are more susceptible to cold stress. Interestingly, the authors also analyzed off-target effects in other members of the annexin family and no off-target mutations are detected, confirming the high specificity of the CRISPR/Cas system in rice.

Genome editing to improve herbicide tolerance

Another important desirable trait that researchers have attempted to introduce in rice lines is resistance to herbicides. Several attempts have been made using transgenic lines (Inui et al, 2001; Zhao et al, 2011; Dong et al, 2017; Fartyal et al, 2018), however, due to the legal and commercial limitations regarding genetically modified organisms, they are moderately successful. The use of genome editing techniques allows the generation of transgene-free edited plants. There are several reports of adaptations of CRISPR/Cas technology to obtain new non-transgenic lines resistant to herbicides. In many cases, this agricultural trait is conferred by point mutations and is best achieved by precise genome editing.

In this sense, a P178S mutation in *Acetolactate Synthase 1* (*ALS1*) lead to herbicide resistance in soybean (Li Z S et al, 2015). ALS1 is a key enzyme for the biosynthesis of branched chain amino acids and is a major target for important herbicides including chlorsulfuron and bispyribac sodium. Sun et al (2016) used the CRISPR/Cas technique with HR repair to replace the wild type *ALS* gene with one carrying two point mutations (W548 L and S627 I) that can lead to herbicide resistance. Two sgRNAs are designed to target the region of interest (about 1 625–1 888 bp of the gene). A single plasmid carrying the Cas9 expression cassette, both sgRNAs and the template for homologous recombination (with the point mutations of interest), is constructed. The template also contains the target site for both sgRNAs, and therefore Cas9 could cut both the genomic DNA and the plasmid with the HR template. The template is flanked by a left arm (100 bp) and a right arm (46 bp) homologous to the target region to improve recombination. In order to provide enough donor fragments for HR, the free DNA donor fragment and the plasmid are co-introduced into rice (Nipponbare) calli through particle bombardment. With this strategy, HR occurs in all the plants analyzed, although in different HR events. Every plant is sequenced to confirm the correct edition in the target region (Sun et al, 2016). It is important to point out that a similar strategy is used previously in maize (Svitashev et al, 2015), but when it is tested in rice, this strategy works much less efficiently, suggesting that a successful strategy in one plant species needs to be modified for other plant species.

Gene replacement can be used to introduce point

mutations. The enzyme 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) has a conserved motif in all plants that is the binding site for its substrate pyruvate and the herbicide glyphosate. A double amino acid substitution (T102I + P106S) in this domain leads to resistance to herbicide (Yu et al, 2015). In rice (Nipponbare), the second exon of *EPSPS* is replaced using CRISPR/Cas9 by designing two sgRNAs to target introns 1 and 2. The second exon with the required mutations flanked by the recognition sites for both sgRNAs is used as donor DNA. With this strategy, exon replacement is obtained in 2% of the analyzed plants. Since sgRNAs are directed to introns, the splicing pattern is also analyzed to make sure that it is not modified by mutations in introns. No alterations in splicing are found (Li J et al, 2016).

Another strategy to induce point mutations through CRISPR/Cas is the fusion of Cas9 to a base editor, such as Petromyzon marinus cytidine deaminase (PmCDA1). This is called target-activation-induced cytidine deaminase (Target-AID). Basically, two mutated forms of Cas9 (dCas9 with no nuclease activity or nCas9 that has nickase activity) are fused to the base editor, so that base edition can be directed to a specific site through sgRNAs. The point mutation C287T of the *ALS* gene endows rice plants with resistance to the herbicide imazamox. Shimatani et al (2017) designed a sgRNA targeting the C287 base, transformed rice (Nipponbare) calli using this system with both dCas9 and nCas9, and selected transformed calli on medium containing imazamox. As a result, 3 and 14 resistant lines from dCas9 and nCas9 transformants are obtained, respectively, and 7 out of the 14 lines obtained with nCas9 contain the expected C287T mutation and no off-target mutations are reported. Moreover, they demonstrated that using this system, point mutations could be transmitted from calli to regenerates and their progenies to obtain selectable marker-free herbicide tolerant plants through segregation (Shimatani et al, 2018).

Another base editor used to create herbicide-tolerant rice plants using CRISPR/Cas is the adenine base editor (ABE), which consists of tRNA adenosine deaminase fused with nCas9. It generates A-T to C-G conversions when directed by sgRNAs. In order to create a suitable ABE system for rice, seven ABE fusion proteins are created that vary in the position of adenosine deaminase and the number and location of the nuclear localization sequences (Li J et al, 2018). These authors chose the most efficient configuration and designed a sgRNA to achieve the point mutation C2186R in the gene coding for acetyl-coenzyme A carboxylase (ACC) in rice (Zhonghua 11) with resistance to herbicides across the aryloxyphenoxypropionate, cyclohexanedione and phenylpyrazoline chemical groups. Analysis of 160 transformed lines shows a mutation efficiency of 20.6% in the target region. Importantly, no off-target mutations are found among the T_1 mutant lines analysed. As expected, rice plants carrying the C2186R mutation are tolerant to the herbicide haloxyfop-R-methyl (Li J et al, 2018).

Yield improvement by genome editing

Grain yield is a complex trait governed by many different factors including agronomical practices and many genes known as quantitative trait loci (QTLs). The most popular method for plant breeding in essence is to generate various QTL combinations and select the elite ones (Shen et al, 2018). Some of these QTLs have been selected as target genes in studies using CRISPR/Cas to obtain new rice lines with improved yield. Li M R et al (2016) mutated the rice (Zhonghua 11) genes *Gn1a*, *DEP1*, *GS3* and *IPA1*, which function as regulators of grain number, panicle architecture, grain size and plant architecture, respectively. The first exon of *Gn1a* and *GS3* and the third exon of *DEP1* and *IPA1* are chosen as targets for the sgRNAs because mutations in those regions have been proven to originate the desired phenotype (higher yield). And the mutation frequencies in T_0 transgenic lines are 42.5%, 67.5%, 57.5% and 27.5% for *Gn1a*, *DEP1*, *GS3* and *IPA1*, respectively. The *Gn1a* mutation increases plant height, panicle size and number of flowers per panicle (about 90% more than the control). The *dep1* mutants show decreased plant height and short panicles (about 20% less than the control), but an increased number of flowers per panicle (about 50% more than the control). Mutant lines of *GS3* show larger grain size and longer awns on the husks. Finally, mutations of *IPA1* result in three phenotypes depending on the nature of the mutation. The sgRNA is designed to mutate the miR156 target site. If the mutation did not originate a frame shift or modify the miR156 target site, the phenotype was the same as the wild type. If the mutation changed the miR156 target site but did not produce a frame shift of the protein, mutant plants had 2–4 tillers, which is fewer than the 7–10 tillers typical of the wild type, but plant height, flower number and panicle length were all increased compared with those of the wild type.

Finally, if the mutation provoked a frame shift in the protein, the mutant plants had a dwarf phenotype with an increased number of tillers (Li M R et al, 2016). Three out of four sgRNAs produce off-target events in the predicted genomic regions. Off-target mutation frequencies are 67% for the sgRNA of *Gn1a*, 47.5% for *IPA1* and 2.5% for *DEP1*. This reinforces the idea that sgRNAs should be carefully designed in order to avoid unwanted genomic modifications. In this way, several bioinformatics tools have been created to design highly efficient and specific sgRNA in order to increase mutation efficiency and avoid unwanted off-target effects (Bae et al, 2014; Doench et al, 2016; Chari et al, 2017; Zhao et al, 2017).

GS3 and *Gn1a* mutations also result in higher grain number or increased grain size (Shen et al, 2018). However, rice yield in those mutants vary depending on the genetic background. Five *japonica* varieties, Nanjing 9108, Wuyunjing 27, Yangjing 4227, Zhejing 22 and Zhejing 88 are chosen for mutagenesis. A single plasmid containing two sgRNAs targeting *GS3* and *Gn1a* is used for *Agrobacterium*-mediated transformation of rice calli. Simple *gs3* and double *gs3gn1a* mutants are obtained for each variety and 10 new genotypes were obtained. Seven out of the ten novel genotypes show decreased grain yields per plant, ranging from 1% to 30% less than wild type. Only three genotypes show grain yields higher than the wild type, with increases of 3%–7%. The explanation is that despite the higher grain number and size of the mutant, some backgrounds show fewer effective tillers and lower grain number per tiller (Shen et al, 2018). Similarly, Zhou et al (2018) observed that mutations in the yield-related genes *GS3*, *GW2* and *Gn1a* result in increased yields per panicle in the varieties Jijing 809 and Liaojing 237, but not in Chuannongxiang jing. Multiplex sgRNA is used to generate double and triple mutants of these genes, and an additive effect of these genes is observed in Jijing 809 and Liaojing 237 (Zhou et al, 2018). Importantly, these authors analyzed the two most likely off-targets of each sgRNA and no mutations at those sites are found. It is important to highlight that analysing mutations in the same genes in different cultivars gives contrasting results. This shows the need to test those mutations in different agronomic cultivars around the globe.

The CRISPR/Cas9-mediated multiplex genome editing system is one of the most attractive characteristics of this technology, especially for modifying QTLs to improve rice yield. In this way, Xu et al (2016) edited three QTLs related to grain weight, *GW2*, *GW5* and *TGW6*. The functions of *GW2*, *GW5* and *TGW6* are well characterized, and null mutations in any of them lead to significant increases in grain weight. Moreover, *gw5tgw6* and *gw2gw5tgw6* mutants have larger grains than the wild type. The double mutant exhibits increases of about 12%, 8% and 13% in grain length, grain width and 1000-grain weight, respectively. The triple mutants display increases of about 25%, 20% and 28% in grain length, grain width and 1000-grain weight, respectively. The results show that this system is well suited for the rapid generation and pyramiding of beneficial alleles in rice.

The possibility of deleting a precise region of a gene with CRISPR/Cas has also been used to increase yield. A 625 bp-deletion in *DEP1* gene confers dense and erect panicles with a higher grain number and lower plant height than the wild type (Huang et al, 2009). Taking this into consideration, Wang et al (2017) designed four sgRNAs (S1–S4) to achieve the same deletion in *indica* rice (inbred line IR58025B). The four sgRNAs are distributed in four different constructs, S1/S4, S1/S3, S2/S4 and S1/S2/S3/S4. Deletion frequencies of upto 21% for a 430 bp target and 9% for a 10 kb target, among 96 T_0 events per construct, are achieved. The *dep1* mutants produced using CRISPR/Cas technology show the desired phenotype (dense and erect panicles and reduced plant height).

Rice heading date, one of the most important agronomic traits determining rice distribution and production, is controlled by both genetic and environmental factors (Matsubara et al, 2014). *Hd2*, *Hd4* and *Hd5* are major genes for negative control of the heading date of rice varieties (Li X F et al, 2015), and mutation of those genes could lead to early-maturing varieties. Different sgRNAs (two for *Hd2* and *Hd4* and one for *Hd5*) are designed by Li et al (2017). Seven rice cultivars, Longdao 16 (*Hd2Hd4Hd5*), Longdao 18 (*Hd2Hd4Hd5*), Daohuaxiang 2 (*Hd2Hd4Hd5*), Songjing 19 (*Hd2Hd4Hd5*), Dongnong 430 (*Hd2Hd4Hd5*), Dongnong 429 (*hd2Hd4Hd5*) and Longqingdao 2 (*hd2hd4Hd5*), were transformed by *Agrobacterium*. A total of 18 independent transgenic lines are generated from the five varieties with genotype *Hd2Hd4Hd5*, among which 14 seedlings show mutations (single-base insertions or deletions) within all three genes, indicating a high efficiency (77.8%) of simultaneous editing of multiple genes. T_1 transgenic lines from the seven varieties are analyzed and nine homozygous T_2 lines are chosen for evaluation. The heading date in all

of the lines is 5–30 d earlier than that in the wild type (Li et al, 2017).

Hybrid rice plays an advantage of 10%–20% over conventional rice in yield, and therefore it has a key role in rice production worldwide. Hybrid lines can be developed using the three- and two-line hybrid breeding systems (Cheng et al, 2007). The two-line breeding system uses either photoperiod-sensitive genic male-sterile (PGMS) or thermo-sensitive genic male-sterile (TGMS) lines as sterility or maintainer lines under restrictive or permissive conditions, respectively. Thus, development of new P/TGMS lines would improve and accelerate the breeding process. Zhou et al (2016) modified the gene *TMS5* by CRISPR/Cas to obtain a new TGMS line. Ten sgRNAs are designed targeting this gene and used to transform rice calli (Zhonghua 11) through *Agrobacerium*. T_0 plants are obtained with mutations in nine out of ten sites, with a homozygous mutation rate of about 32%. Among T_0 plants, 30%–85% were pollen-sterile individuals under restrictive conditions. To obtain TGMS plants, T_0 sterile plants are treated at a low temperature and transgene-free TGMS lines are obtained in T_1 plants. Furthermore, the most efficient sgRNA is chosen to transform 11 cultivars to obtain TGMS plants, including ten *indica* cultivars (Zhenshan 97B, Zhongzhe B, Tiangfeng B, Yixiang B, Re B, Huahui B, Huanong B, Yuejingsimiao, Yuenongsimiao and Wushansimiao) and one *japonica* cultivar GAZ. In this case, the mutation frequency ranges from 72% to 100% with a homozygous mutation rate of 11%–54%. Mutant transgene-free plants are obtained in T_1 generation and the mutations are stable in T_2 and T_3 generations. To obtain TGMS lines, these lines are treated with different temperature gradients and found that fertility is gradually reduced with increasing temperature. The use of CRISPR/Cas to produce new TGMS lines can facilitate the exploitation of heterosis in breeding process. Moreover, this technology can be applied potentially for breeding other hybrid crops not only rice.

Genome editing application in rice quality

Another goal of rice improvement is to create new varieties with increased nutritional and commercial value. Of particular interest is the modification of starch and amylose content. A cereal grain higher in amylose is a good source of resistant starch, which is a special kind of starch that is not digested in the stomach or small intestine, but passes directly to the large intestine. Resistant starch helps to improve human health and reduce the risk of non-infectious diseases (Regina et al, 2006). Amylose and resistant starch contents can be increased by modifying the starch branching enzymes (SBEs). *SBEIIb* is mainly expressed in rice grains, which is why it is chosen as a target for CRISPR/Cas mutation. After *Agrobacterium*-mediated transformation of rice (Kitaake) calli, 22 of 30 transgenic plants show mutations in the expected region, with frequencies of 6.7%, 36.6% and 26.7% for heterozygous, bi-allelic and homozygous lines, respectively. No off-target effects are detected in the predicted sites. After the generation of transgenic-free mutant lines through segregation, *sbeII* mutants show significantly increased amylose and resistant starch contents (upto 25.0% and 9.8%, respectively) (Sun et al, 2017).

Amylose content is also the most important quality indicator in rice, especially for cooking and eating quality. The quality of some rice varieties, especially *indica* hybrids, is considered poor in some markets owing to their high amylose content. In this case, reducing the amylose content to obtain glutinous rice is a highly desirable characteristic. A single dominant gene called *Waxy* controls the amylose content in rice. Therefore, in order to obtain a loss-of-function mutant through CRISPR/Cas, a sgRNA targeting the first exon of the *Waxy* gene is designed by Zhang et al (2018). Following *Agrobacterium*-mediated calli transformation of two cultivars (Xiushui 134 and Wuyunjing 7), a high mutagenesis efficiency (82%–87%) is observed in T_0 transformants. About 4%–15% of the mutants were homozygous. Interestingly, no off-target mutations are observed in any of the mutants analyzed. Analysis of agronomic traits in T_1 generation plants shows no differences between wild type and *waxy* mutants regarding plant height, grain number per panicle, panicle number per plant, yield per plot, grain width, grain length and 1000-grain weight. The amylose content is significantly lower (85%) in *waxy* mutants than in the wild type plants (Zhang et al, 2018).

Rice bran oil (RBO) is a commercial derivative of rice that is produced and consumed mainly in Asian countries. RBO contains components with valuable health-promoting properties, one of which is oleic acid, which helps prevent lifestyle diseases. Modifications in fatty acid synthesis pathways could lead to higher levels of oleic acid and more valuable RBO. The enzyme fatty acid desaturase 2 (FAD2) is responsible for the conversion of oleic acid to linoleic acid. The rice genome has four *FAD2* genes, of which *FAD2-1*

is the most highly expressed in rice seeds. Thus, the knockout of *FAD2-1* by CRISPR/Cas could lead to a variety with high oleic acid content. Through *Agrobacterium*-mediated transformation of rice (Nipponbare) calli, six independent mutant lines are regenerated. After evaluating the progeny to confirm the mutation, fatty acid profiles of the T_2 lines are analyzed. In *fad2-1* homozygous mutants, the oleic acid content increases to more than twice of that in wild type, and linoleic acid is not detected (Abe et al, 2018).

Cadmium (Cd) is highly toxic heavy metal for living organisms. Rice grains with excesive Cd content are a serious threat for people who consume rice as a staple food. Persistent intake of Cd could lead to different health problems (Bertin and Averbeck, 2006; Clemens et al, 2013). The development of elite rice cultivars with low Cd accumulation is very important, especially for *indica* varieties, which can accumulate more Cd than *japonica* ones. Tang et al (2017) created a new *indica* rice line with low Cd accumulation by mutating the metal transporter gene *NRAMP5* using CRISPR/Cas9 system. This transporter mediates the root uptake of Cd, and therefore mutating this gene causes a great reduction in Cd content. Two sgRNAs are designed to target the ninth exon of the gene. *Agrobacterium*-transformation of embriogenic calli leads to a mutation efficiency of 82.4% for Huazhan and 80% for Longke 6385. No off-targets effects are observed. Five (three from Huazhan and two from Longke 6385) transgene-free homozygous knockout lines are obtained in T_1 generation by segregation. *nramp5* lines show less Cd accumulation (0.05 mg/kg) when grown on Cd-contaminated paddy fields compared to the wild type (0.33–2.90 mg/kg). Moreover, mutant plants show no differences in agronomic traits such as grain yield, straw weight or grain quality compared with wild type plants.

Genome editing application in nutrient use

As mentioned in previous sections, the CRISPR/Cas system can accelerate breeding processes that would otherwise take years using traditional crossing/ backcrossing techniques. An example of this is the introduction of an elite allele into commercial cultivars. For instance, a single nucleotide polymorphism in the nitrate transporter *NRT1.1B* gene is responsible for improved nitrogen use efficiency in *indica* rice. Therefore, the replacement of this gene could lead to commercial cultivars with improved nitrogen use efficiency. Li J Y et al (2018) employed the strategy described earlier (Sun et al, 2016) with some modifications to replace the *japonica* allele with the *indica* allele. First, the two sgRNAs target different strands of DNA to minimize the non-homologous sequences when released from the vector. Second, the PAM site is mutated in the template sequence to prevent further cuts after HR is successful. Finally, a longer right arm is used to enhance HR frequency. With this improved strategy, the precise replacement of the allele, either homozygous or heterozygous, is obtained in 6.72% of the transformed plants. Partial HR and reverse complementation are also observed, but no off-target mutations are found in the plants analyzed (Li J Y et al, 2018), which demonstrated that CRISPR/Cas technology can be used for efficient allelic replacement by HR in rice in one generation, thereby accelerating the crop improvement process.

PERSPECTS

Since the CRISPR/Cas genome editing system was first implemented in 2013, its use and efficiency for site-directed mutation have been tested in plants. This system has become the most common tool for functional and/or agronomic studies in different plant species, especially in rice, which is not only very important at the agronomic level, but is also a model species for physiological and molecular studies in monocots. The growing interest and constant search for new applications has led to the generation of new knowledge and improvements that facilitate the application of this technology in rice and other crops of agronomic interest. The research demonstrates the wide variety of applications of this technology: the generation of small InDels that cause mutation by frame shift changes, large gene deletions, the precise edition of certain bases to generate point mutations using the base-editing systems coupled to CRISPR/Cas and gene substitution by homologous recombination at specific sites in the genome. Even the generation of a collection of mutants in more than 12 000 genes is possible thanks to this technology. This technology and the growing advances in massive sequencing techniques have made possible the creation of new rice lines with specific site directed mutations without off-target mutations. More importantly, because of the strict regulation of transgenic organisms, this technique allows the generation of transgene-free mutant plants. Altogether, this technique facilitates not only the

analysis of the function of different genes, but also the process of genetic improvement of this important species. It is because of all these advantages that this technology is preferred by researchers dedicated to genetic improvement of rice worldwide.

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