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REVIEW

Targeted genome modification technologies and their applications in crop improvements

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Abstract Recent advances in genome engineering indicate that innovative crops developed by targeted genome modification (TGM) using site-specific nucleases (SSNs) have the potential to avoid the regulatory issues raised by genetically modified organisms. These powerful SSNs tools, comprising zinc-finger nucleases, transcription activator-like effector nucleases, and clustered regulatory interspaced short palindromic repeats/CRISPR-associated systems, enable precise genome engineering by introducing DNA double-strand breaks that subsequently trigger DNA repair pathways involving either non-homologous end-joining or homologous recombination. Here, we review developments in genome-editing tools, summarize their applications in crop organisms, and discuss future prospects. We also highlight the ability of these tools to create non-transgenic TGM plants for next-generation crop breeding.

Keywords Targeted genome modification · Site-specific nucleases (SSNs) · ZFNs · TALENs · CRISPRs · Crop improvement

Introduction

Although targeted genome modification (TGM) in plants was first demonstrated in the late 80s, it remained for a long time far from routine because of its low efficiency (Paszkowski et al. 1988). Very recently, revolutionary advances in the engineering of site-specific nucleases (SSNs) have allowed progress to be made in the precise manipulation of the genomes of model plants and important crops (Curtin et al. 2012; Voytas 2013). Up to now, waves of innovation have involved three SSNs: zinc-finger nucleases (ZFNs), transcription activator-like nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas-mediated RNA-guided DNA endonucleases (Gaj et al. 2013). ZFNs, involved in the first wave of SSN innovation (Carroll 2011), consist of a zinc-finger DNA-binding domain (DBD) fused to the non-specific cleavage domain of *Fok*I, and were developed more than a decade ago. Although considerable achievements have been made with it, this tool remains difficult to use for many reasons, such as the difficulty in designing the constructs, the context dependence of the repeat units, frequent off-target effects and limited target sites (DeFrancesco 2012). In 2009, two groups independently discovered the code for transcription activator-like effectors (TALE)-DNA recognition (Boch et al. 2009; Moscou and Bogdanove 2009), and TALENs emerged as an alternative to ZFNs and prompted the second revolutionary wave of SSN-mediated TGM (Cermak et al. 2011; Li et al. 2011). Like ZFNs, TALENs consist of customizable TALE-DBDs with *Fok*I cleavage domains. However, TALENs work more efficiently than ZFNs, and are much easier and cheaper to make (Bogdanove and Voytas 2011; DeFrancesco 2012; Joung and Sander 2012). In 2011, the journal *Nature Methods* chose genome-editing techniques, comprising ZFNs, TALENs and meganucleases, as "the method of the year for 2011" (Baker 2012). Then, at the end of 2012, TALENs were crowned one of the 10 breakthroughs of the year by the journal *Science* (Alberts 2012). In the same year, the first report on a new genome

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engineering technique based on the bacterial endonuclease Cas9 appeared (Jinek et al. 2012). By now, in late 2013, the RNA-based CRISPR/Cas system has been successfully used in a variety of organisms, including plants (Pennisi 2013).

Current approaches to accelerating crop improvement mainly depend on conventional and transgenic breeding methods. Although it has been used in many crop species for thousands of years, conventional breeding is restricted by a declining genetic base dependent on existing natural allelic variations such as germplasm collections and randomly induced variants generated by physical or chemical mutagens. On the other hand, transgenic breeding usually results in the production of genetically modified crops (GM crops) and these have made a considerable contribution to securing food supplies since they were first commercially grown in 1996. However, GM crops generally carry foreign genes inserted at random in the genome, and their commercialization is frequently prevented by public concern about health and environmental safety and by complex regulatory requirements. Fortunately, the newly developed techniques of TGM based on SSNs are attractive alternative approaches to the precise manipulation of genomes that avoid some of these difficulties.

The core principle of genome engineering based on SSNs relies on DNA repair mechanisms triggered by DNA double-strand breaks (DSBs) introduced by SSNs at given genomic sites. The DSBs can be repaired by either non-homologous end-joining (NHEJ) or homologous recombination (HR) (Wyman and Kanaar 2006). By these processes, SSNs can generate gene knockouts, replacements, insertions, and chromosome rearrangements (Chen and Gao 2013; Curtin et al. 2012). The precision of the modification procedures should overcome some of the constraints and uncertainties associated with conventional and transgenic breeding systems, and permit the new TGM crops to circumvent the regulatory mechanism aimed at GMOs (Lusser et al. 2012).

This review focuses on the development of the three SSN-based techniques together with their applications to crop plants. We also discuss aspects of the regulatory oversight of TGM crops developed using these SSNs, and highlight the potential applications of SSNs in crop improvement.

SSN-based TGM technologies

ZFNs: the pioneer of SSNs

More than a decade ago, a new technology involving ZFNs provided a way to target specific genes. The Cys_2-His_2 zinc finger (ZF) domain is the most abundant DNA-binding motif in eukaryotes. Each ZF, consisting of approximately 30 amino acids that fold into a ββα configuration, is stabilized by a zinc ion. The ZF can recognize and bind to a specific 3-bp DNA sequence by inserting an α-helix into the major groove of the DNA double helix (Pabo et al. 2001). ZFNs are generated by fusing an artificial DBD consisting of a tandem array of ZFs to the non-specific DNA cleavage domain of the FokI restriction endonuclease (Fig. 1) (Durai et al. 2005). A typical ZF DBD is composed of two arrays of 3 or 4 individual ZFs each (two ZFAs) that can bind to a 9- or 12-bp target sequence. The cleavage domain functions typically as a dimer, and the pair of ZFAs are designed to bind sequences within a distance of 5–7 nucleotides from each other; the result is an enzyme capable of targeting a unique DNA sequence and able to induce targeted DSBs (Durai et al. 2005).

Zinc-finger nucleases can be designed to recognize defined genomic sites by altering and combining ZFs. A variety of strategies have been explored to create specific ZFNs efficiently. One is known as "modular assembly", a method of engineering multi-finger arrays that treats individual fingers as independent units (Bae et al. 2003; Wright et al. 2006). But the success rate of ZFNs made by this method has been reported to be low due to its failure to account for the context effects of zinc-finger domains in an array. To overcome this context dependence, a selection-based method, oligomerized pool engineering (OPEN) is used in TGM. The OPEN strategy employs a collection of zinc-finger pools, each consisting of a small number of different fingers designed to bind to a particular 3-bp unit, and an in vivo-based selection method (Maeder et al. 2008). The labor and expertise required to screen combinatorial libraries have limited its broad adoption, and an easier and effective alternative for making ZFNs, called context-dependent assembly (CoDA) has been developed (Sander et al. 2010). Using archives of preselected two-finger units that are validated, CoDA can rapidly assemble ZF arrays using simple cloning strategies. With the current archive of CoDA units, a potential ZFN target site can be found approximately once in every 200 bp of random sequence by OPEN, and one site in 500 bp by CoDA (Maeder et al. 2008; Sander et al. 2010). ZFNs have been employed to modify many plant genes in *Arabidopsis* (Osakabe et al. 2010; Zhang et al. 2010), petunia (Marton et al. 2010), tobacco (Maeder et al. 2008; Townsend et al. 2009), soybean (Curtin et al. 2011), and corn (Shukla et al. 2009). However, the use of ZFNs is still challenging for most molecular genetic laboratories due to the difficulty of producing them, their high cost, and their modest efficacy in many applications.

TALENs: the core genomic cruise missile of SSNs

Since the discovery of "the code" connecting the repetitive regions of transcription activation-like effector proteins

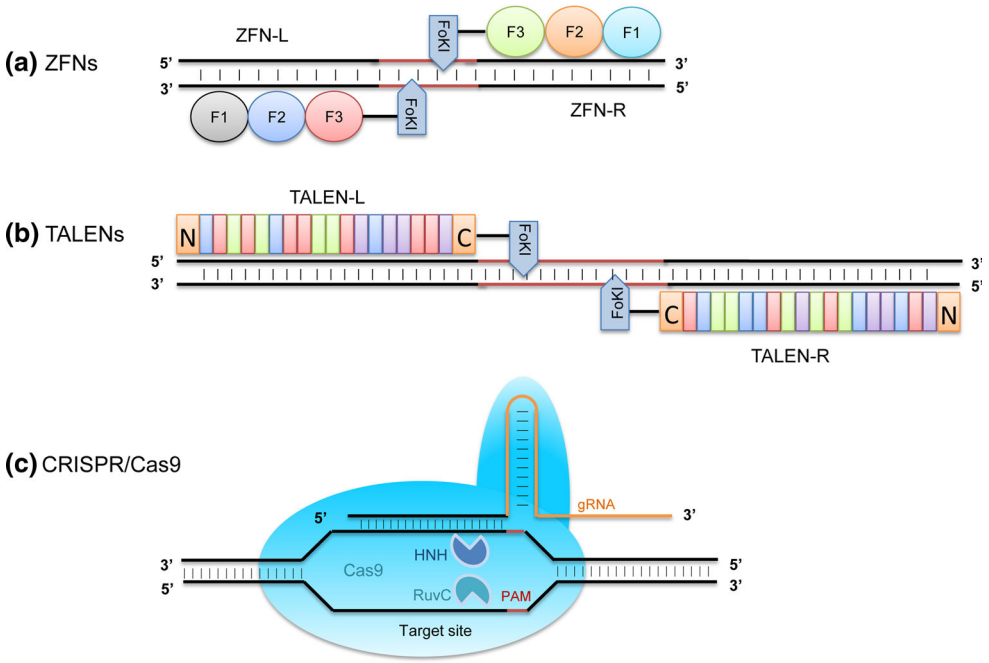

Fig. 1 Schematic of the three site-specific nucleases. **a** Zinc finger nucleases (ZFNs). **b** Transcription activator-like effector nucleases (TALENs). **c** The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated system. ZFNs (**a**) and TALENs (**b**) consist of DNA binding domain (DBD) fused to FokI nuclease. Each zinc finger module recognizes and binds to three nucleotides of the targeted sequence; each TALE module specifically binds one nucleotide. Both depend on dimerization of FokI to cleave the target sequence. The CRISPR/Cas system (**c**) consists of a sgRNA and the Cas9 nuclease. One nucleotide of the sgRNA interacts with one nucleotide of the DNA target site. Cas9 unwinds the DNA duplex and cleaves both strands upon recognition of a target sequence by the gRNA, but only if the correct PAM is present at 3′ end

with the DNA bases to which they bind (Boch et al. 2009; Moscou and Bogdanove 2009), TALENs have become the current reagent of choice for efficiently modifying eukaryotic genomes in a targeted fashion (Baker 2012).

Like ZFNs, TALENs are composed of an engineered array of DBDs fused to a non-specific *Fok*I nuclease domain (Fig. 1) (Christian et al. 2010). TALEs, secreted by the plant pathogenic bacterial genus *Xanthomonas*, are major virulence factors which cause disease in plants by activating the transcription of specific target genes (Boch and Bonas 2010). TALEs have specific structures, including N-terminal secretion and translocation signals, C-terminal nuclear localization signals (NLS) and an acidic transcription-activation domain (AD), and a central DBD (Boch and Bonas 2010; Bogdanove et al. 2010). The central DBD typically consists of 14–20 tandem arrays of highly conserved 34 amino acid repeats. The amino acid sequences of the repeats are nearly identical except for the di-residues at positions 12 and 13, designated "repeat variable di-residues" (RVDs). The crystal structures of these effector proteins have revealed that the second amino acid of the RVD (position 13) mediates specific recognition of the sense strand DNA base, while the first amino acid (position 12) helps to stabilize the repeat structure (Deng et al. 2012; Mak et al. 2012). The following recognition preferences have been experimentally demonstrated for DNA recognition by TALEs: HD = C, NG = T, NI = A, NN = G or A (Boch et al. 2009; Moscou and Bogdanove 2009). Recent studies show that NK and NH have higher specificity for guanine (G) than NN does (Cong et al. 2012; Streubel et al. 2012). By employing this simple RVD-nucleotide code, TALENs can be customized by assembling them according to the desired target sequence. Unlike ZFNs, which only recognize a limited number of target sites in the genome, TALENs can easily be designed to target any recognition site provided only that there is a thymine (T) before the first nucleotide of the target site.

Although TALEs have the advantage of greater flexibility, the long and extensive repetitive nature of their DBDs makes it a major challenge to construct engineered TALENs. Nevertheless, several TALEN assembly methods, such as standard cloning assembly methods (Huang et al. 2011; Reyon et al. 2012a; Sander et al. 2011), Golden Gate assembly methods (Cermak et al. 2011; Li et al. 2011; Weber et al. 2011) and solid-phase assembly methods (Reyon et al. 2012b), have been developed. Because the costs of the Golden Gate method are low, it is easily manipulated and it is suitable for small scale research requirements, most TALENs targeting plant genes have been constructed by that method, which uses a ligation-

based strategy (Cermak et al. 2011; Li et al. 2011; Morbitzer et al. 2011; Sander et al. 2011; Weber et al. 2011). It employs Type IIS restriction enzymes to create multiple sticky ends, cloning is carried out by digestion and ligation in the same reaction mixture, and researchers can easily ligate up to 10 TALE repeats in one reaction. In the 4 years since the technique was developed, TALEN technology has been adopted in many organisms including plants such as tobacco (Zhang et al. 2013), rice (Li et al. 2012; Shan et al. 2013a), *Brachypodium* (Shan et al. 2013a), barley (Wendt et al. 2013), and *Arabidopsis* (Cermak et al. 2011; Christian et al. 2010, 2013).

CRISPRs: new SSN troops

Innovation in the TGM field has proceeded with such rapidity that before one had even recovered from the shock induced by TALENs, the extraordinary CRISPR/Cas system, which generates DSBs in an even more efficient, simpler, and quicker way, had opened another exciting chapter in the ever-expanding tale of genome engineering (Barrangou 2012; Pennisi 2013).

Clustered regularly interspaced short palindromic repeats and CRISPR-associated systems (Cas) provide RNA-mediated adaptive immunity against invading plasmids and viruses, and are widespread in bacteria and archaea (Barrangou 2013). Based on their components and sequences, three CRISPR systems have been identified (Barrangou 2013). Type I and type III involve multiple proteins forming a large functional Cas complex, while the type II CRISPR system, from *Streptococcus pyogenes*, which relies on a single endonuclease, Cas9, has been adopted for genome engineer. In this system, when short DNA fragments from a virus or plasmid are integrated into a CRISPR locus, segments of the invader's DNA are converted into CRISPR RNAs (crRNAs) and in turn anneal to the trans-activating crRNA (tracrRNA) to form a dual-RNAs structure. The dual-RNA structure guides Cas9 to a region called the protospacer in the DNA of the invader. The Cas9 then cleaves the protospacer DNA on both strands—the HNH nuclease domain cleaves the complementary strand and the RuvC-like domain cleaves the non-complementary strand (Fig. 1).

Jinek et al. have demonstrated targeted cleavage in vitro using Cas9 and a single guided RNA (sgRNA) molecule, which was generated by annealing a dual-tracrRNA: crRNA (Jinek et al. 2012). This milestone achievement has provided the foundation for CRISPR genome engineering. At the beginning of 2013, two groups independently demonstrated targeted DNA cleavage in human and mouse cells by CRISPR/Cas (Cong et al. 2013; Mali et al. 2013). The only constraint on CRISPR/Cas is that the recognition sites need always to be preceded by a 5′-NGG protospacer-adjacent motif (PAM). Only a single customized gRNA, encoded by a sequence of approximately 100 nt is required to target a specific sequence, and Cas9 does not have to be reengineered for each new target site. The CRISPR system is therefore much more straightforward than ZFNs or TALENs. Since last year, CRISPRs have been used to delete, add, activate and suppress targeted genes in many organisms (Pennisi 2013), demonstrating the broad applicability of this technology. The CRISPR system has been applied in numerous plant species including the model plants *Arabidopsis* (Feng et al. 2013; Li et al. 2013; Mao et al. 2013) and tobacco (Li et al. 2013; Nekrasov et al. 2013), and the crop plants rice (Feng et al. 2013; Jiang et al. 2013; Mao et al. 2013; Miao et al. 2013; Shan et al. 2013b), wheat (Shan et al. 2013b) and sorghum (Jiang et al. 2013).

Table 1 Comparison of ZFN, TALEN, and CRISPR-mediated genome engineering

	ZFN	TALEN	CRISPR
Binding principle	Protein-DNA	Protein-DNA	RNA-DNA
Core components	ZFA-FokI fusion protein	TALE-FokI fusion protein	sgRNA and Cas9
Work mode (pair)	Pair	Pair	No
Design	Moderate	Easy	Very easy
Construction	Difficult	Easy	Very easy
Time for construction (days)	5–7	5–7	1–3
Cost	High	Moderate	Low
Efficiency	Variable	High	High
Off-target rate	High but variable	Low	High
Length of target sequence	~18- to 24-bp (including 5–7 bp spacer)	~50–60 bp (including 14–18 bp spacer)	~20 bp

Each of the three SSNs has its advantages and disadvantages. A brief comparison between them is presented in Table 1.

Categorization and applications of SSN-based TGM in crop plants

Site-specific nuclease-mediated genome modification depends on the introduction of DSBs at target sites that are repaired by either NHEJ or HR DNA repair machinery (Fig. 2) (Wyman and Kanaar 2006). NHEJ, the simplest error-prone repair process, which often results in small

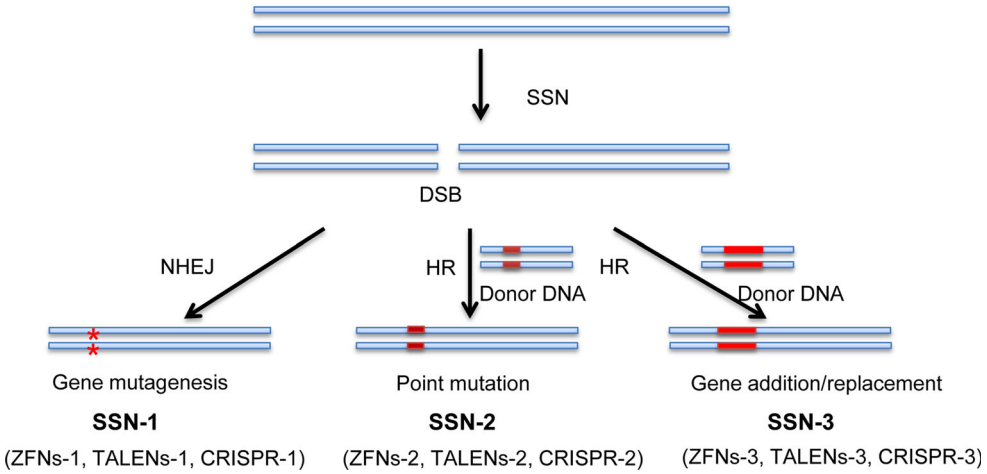

Fig. 2 Classification of site-specific nuclease (SSN) techniques. Double-strand breaks (DSBs) induced by ZFNs, TALENs and CRISPRs activate DNA repair mechanisms involving either non-homologous end-joining (NHEJ) or homologous recombination (HR). With SSN-1, small insertions or deletions can be generated through NHEJ; SSN-2 produces point mutations via HR. In SSN-3, transgene insertions are generated by HR

deletions and/or insertions (indels), can produce gene knockouts by generating frame shift mutations in coding sequences. Alternatively, in the presence of a homologous donor DNA spanning the DSB, the HR repair pathway can be activated, and a targeted gene replacement, integration, or addition can result.

Depending on the level of integration of the recombinant DNA into the plant genome, along with consideration of the regulatory oversight that is applicable, SSN techniques can be divided into three categories (Fig. 2) (Lusser et al. 2012; Podevin et al. 2013). SSN-1 includes the ZFN-1, TALEN-1 and CRSIPR-1 techniques. In this group, SSNs are delivered into plant cells by themselves and create site-specific DSBs which are repaired by NHEJ. SSN-1 leads to frame-shift mutations due to small insertions or deletions (indels). SSN2 includes the ZFN-2, TALEN-2, and CRISPR-2 techniques. In this group, SSNs are delivered into plant cells together with a short DNA repair template, consisting of a DNA sequence homologous to the target site except for one nucleotide. SSN-2 generates the desired site-specific point mutations by HR. SSN-3 includes the ZFN-3, TALEN-3, and CRISPR-3 techniques. In these, SSNs are delivered into plant cells along with a transgene (insertion) which has homologous sequences flanking the target site at both ends. SSN-3 generates site-specific DSB which are repaired by HR. This technique can be used for targeted transgene insertion, gene replacement and gene stacking at predetermined sites.

Up to now, although most TGM studies have focused on model species, an increasing amount of work has been reported on crop plants. Here, we briefly review the achievements in crop plants made possible by SSN technologies (Table 2).

The first successful case of ZFN1-mediated gene knockout of an endogenous target was with the tobacco acetolactate synthase gene (*ALS*) named SuRA (Maeder et al. 2008). Later, seven of nine endogenous soybean genes were targeted by the ZFN-1 technique (Curtin et al. 2012). Since soybean is paleopolyploid with approximately 75 % of its genes duplicated, this finding indicates that ZFN1-based mutagenesis is an efficient method for making mutations in duplicate genes.

The use of TALEN-1 to induce TGM in endogenous targets has been reported in rice. First Li et al. introduced a mutation in the promoter region of the sucrose-efflux transporter gene, *OsSWEET14* (Li et al. 2012). The consequence of the mutation was increased resistance to bacterial blight. Later, we achieved in our laboratory large scale highly efficient targeted gene modification by the TALEN-1 technique in rice and *Brachypodium* (Shan et al. 2013a). Wendt et al. (2013) have targeted the region that contained a group of regulatory motifs in the promoter of a barley phytase gene.

The first application of CRISPR-1 in crops was reported by our group. Four rice genes and one wheat gene were targeted with high efficiency (Shan et al. 2013b). Stably mutated plants were obtained in the T0 generation, with biallelic mutants of OsPDS showing the typical albino and dwarf phenotype (Fig. 3). In the same journal issue, two other groups reported work using CRISPR-1 in *Arabidopsis* and tobacco, respectively (Li et al. 2013; Nekrasov et al. 2013). Subsequently, there have been several reports describing the production of site-specific mutations using CRISPR-1 in rice (Feng et al. 2013; Jiang et al. 2013; Mao et al. 2013; Miao et al. 2013; Shan et al. 2013b) and sorghum (Jiang et al. 2013). This work has mainly focused on genes which, when mutated, have obvious growth phenotypes. The SSN-1 techniques can also be designed to introduce two DSBs, in which case deletions of the regions between two target sites can be obtained. Our group first reported a 1.3 kb fragment deletion in the *OsBADH2* gene (Shan et al. 2013a). SSN-3 techniques have been used to replace targeted nonfunctional reporter genes and endogenous genes. For example, high-frequency ZFN-stimulated

Table 2 Applications of SSNs (ZFNs, TALENs, and CRISPRs) in crop plants

Outcome	Targeted gene names	SSN techniques	Crop species	Modification type	References
Gene mutagenesis	*DCL, DCL4b, RDR HEN*	ZFN-1	Soybean	NHEJ	Curtin et al. (2011)
	Promoter of *OsSWEET11*	TALEN-1	Rice	NHEJ	Li et al. (2012)
	OsDEP1, OsBADH2, OsCKX2, OsSD1	TALEN-1	Rice	NHEJ	Shan et al. (2013a)
	Promoter of *HvPAPhy_*	TALEN-1	Barley	NHEJ	Wendt et al. (2013)
	OsPDS,OsBADH2, Os02g23823, OsMPK2, TaMLO	CRISPR-1	Rice	NHEJ	Shan et al. (2013b)
	CAO1, LAZY1	CRISPR-1	Rice	NHEJ	Miao et al. (2013)
	ROC5, SPP, YSA	CRISPR-1	Rice	NHEJ	Feng et al. (2013)
	mDeRED,	CRISPR-1	Sorghum	NHEJ	Jiang et al. (2013)
	Promoters of *OsSWEET14* and *OsSWEET11*	CRISPR-1	Rice	NHEJ	Jiang et al. (2013)
	OsMYB1	CRISPR-1	Rice	NHEJ	Mao et al. (2013)
Gene addition	*IPK1*	ZFNs-3	Corn	HR	Shukla et al. (2009)
	OsPDS	CRISPR-3	Rice	HR	Shan et al. (2013b)

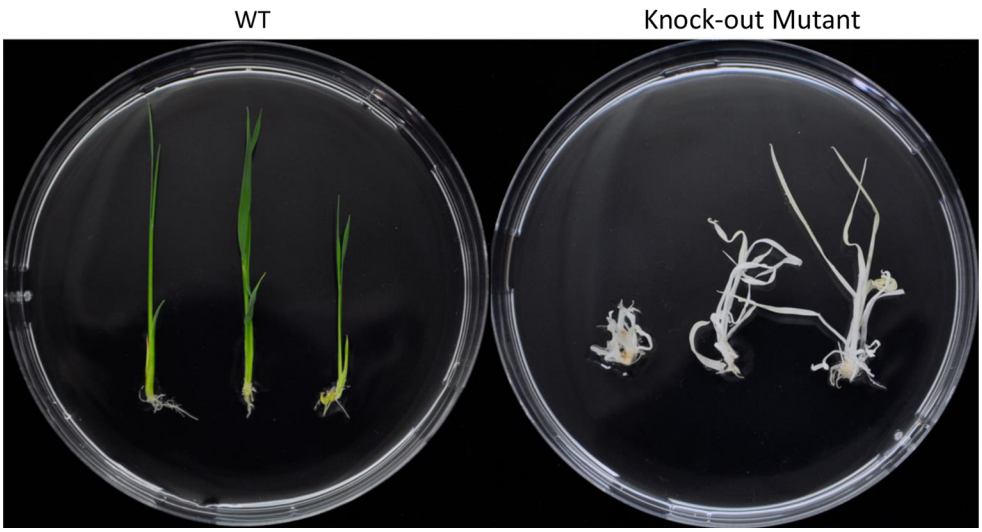

Fig. 3 Dwarf and albino phenotype of a rice *OsPDS* knockout produced by the CRISPR-1 technique

gene targeting of the tobacco acetolactate synthase loci (*ALS*, *SuRA,* and *SuRB*) was reported by Townsend (Townsend et al. 2009). Donor templates with a missense mutation that confers resistance to one or more herbicide, were electroporated into tobacco protoplasts along with ZFNs. Herbicide-resistance mutations were introduced into the *SuR* loci in 0.2–4 % of randomly selected resistant calli cells. TALEN mediated gene replacement was also carried out on the same *ALS* gene in tobacco protoplast (Zhang et al. 2013). When TALEN was co-transformed with a 322 bp donor DNA carrying a 6-bp variant of *SurA* or *SurB*, targeted gene replacements were achieved in about 4 % of calli.

Transgene addition has been demonstrated in corn and tobacco by employing ZFN-3 through the HR pathway. In 2009, Shukla et al. (2009) introduced an important agronomic trait by targeted modification of the *IPK1* gene in corn. They showed that simultaneous expression of ZFN and a simple heterologous donor molecule could lead to precise targeted addition of the *PAT* herbicide-tolerance gene at the intended *IPK1* locus. These mutants not only displayed herbicide tolerance due to expression of *PAT*, but also, as expected, accumulated high levels of phytate and low levels of inorganic phosphate because *IPK1* expression was abolished (Shukla et al. 2009). Similarly, Cai et al. (2009) delivered a Ti plasmid harboring both the ZFNs and a donor DNA construct comprising a PAT cassette flanked by short stretches of homology to the endochitinase locus, and obtained a frequency of up to 10 % of targeted, homology-directed transgene integration (Cai et al. 2009).

Our group demonstrated the introduction of a single-stranded oligo nucleotide with a *Kpn*I + *Eco*RI site into OsPDS via CRISPR in rice protoplast, which proved that HR-dependent targeted gene replacement could be achieved in rice (Shan et al. 2013b). No examples of the application of TALEN-3-mediated gene integration have yet been reported in crop plants.

A promising platform for creating TGM crops

Although GMO crops have been in commercial use for nearly 20 years and have greatly alleviated the food crisis brought about by the ever-growing world population, the public, especially in Europe, is still far from accepting GM crops because of health and environmental considerations. In contrast to classical transgenic breeding, the precise manipulation of genomes by SSN techniques may overcome some of the constraints associated with transgene-based breeding techniques. Furthermore, SSN techniques should avoid introducing novel genetic elements and proteins, and therefore have the advantage over transgenic procedures of reducing potential risks and thus of enjoying greater public acceptance (Lusser et al. 2012; Podevin et al. 2013). SSNs permit more precise and predictable modification of plant genomes, and therefore offer the prospect of major applications in crop improvement. The SSN-1 and SSN-2 techniques can introduce subtle modifications, such as small deletions and single-base substitutions of target genes. The final plants derived by SSN-1 and SSN-2 techniques are similar to natural variants, or those produced by physical or chemical mutagenesis in conventional breeding. SSN-3 techniques allow scientists to insert foreign genes at predefined sites, and this site-specific gene addition should prevent the "position effects" associated with random insertion of genes into plant genomes. The delivery of SSN DNAs using *Agrobacterium* or other delivery methods requires that the SSN DNAs integrate at different loci from the target loci; hence these foreign SSN DNAs can be easily eliminated from the plant genome during the segregation and recombination accompanying sexual reproduction. The final plants generated by SSN-1 or SSN-2 should fall outside the existing definitions and regulation affecting GM crops (Lusser et al. 2012; Podevin et al. 2013). Thus, SSN techniques have great advantages over existing transgenic breeding techniques.

The results of a written survey of a number of plant biotech companies revealed that ZFN technology had been used in breeding of maize, oilseed rape, and tomato (Lusser et al. 2012). Also, Dow AgroSciences has received assurance from the US Department of Agriculture (USDA, Washington, DC) that their genetically modified corn developed by the ZFN technique will not require regulatory oversight (Waltz 2012). This is the first time that the SSN techniques and TGM crops have entered the public arena. Of course, risk assessment will be required before commercialization to ensure food and environment safety.

With regard to plants generated by SSN-2 and SSN-3, the USDA has initiated a case-by-case policy. However, the SSN-3 technique still presents a major problem because of the inserted transgene, even though the targeted nature of the transgene insertion may minimize the hazards involved: most countries will still consider the final products derived of this technique to be GMOs.

Conclusions

In summary, the tremendous progress in site-specific techniques offers a more precise route to crop improvement. Together with the ever-increasing number of sequenced crop plant genomes, and more effective transformation systems, SSN-based TGM crop breeding offers great promise of creating non-transgenic crops with predetermined traits. We believe that these techniques are among the most promising new biotechnology tools for plant breeding.

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