

Review article

Genome engineering via TALENs and CRISPR/Cas9 systems: challenges and perspectives

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Summary

The ability to precisely modify genome sequence and regulate gene expression patterns in a site-specific manner holds much promise in plant biotechnology. Genome-engineering technologies that enable such highly specific and efficient modification are advancing with unprecedented pace. Transcription activator-like effectors (TALEs) provide customizable DNA-binding modules designed to bind to any sequence of interest. Thus, TALEs have been used as a DNA targeting module fused to functional domains for a variety of targeted genomic and epigenomic modifications. TALE nucleases (TALENs) have been used with much success across eukaryotic species to edit genomes. Recently, clustered regularly interspaced palindromic repeats (CRISPRs) that are used as guide RNAs for Cas9 nuclease-specific digestion has been introduced as a highly efficient DNA-targeting platform for genome editing and regulation. Here, we review the discovery, development and limitations of TALENs and CRISPR/Cas9 systems as genome-engineering platforms in plants. We discuss the current questions, potential improvements and the development of the next-generation genome-editing platforms with an emphasis on producing designer plants to address the needs of agriculture and basic plant biology.

Introduction

The ability to modify genomes in a site-specific manner has the potential to transform plant biological research and biotechnology. Precise genome modification can provide control over genetic information and will be used to address basic biological questions and to accelerate biotechnology (Voytas, 2013). Thus, genome-editing tools will facilitate functional genomic studies to address basic questions relating to plant growth, development and responses to the environment. Linking genotype to phenotype is still a primary challenge in functional genomics. Knowledge about gene function and regulation will have broad applications ranging, for example, from engineering crops for improved traits to treat genetic diseases (Liu *et al.*, 2013). With the great advancements in sequencing technologies, the generation of sequence information is highly tractable, but understanding gene function is still a challenge.

Molecular biologists have attempted to study how a particular genotype influences a phenotype by developing reverse genetics approaches including targeted gene inactivation by harnessing homology directed repair processes (Adams and Sekelsky, 2002). The gene targeting technique pioneered by Mario Capecchi, Martin Evans and Oliver Smithies was very efficient in mouse embryonic stem cells (ESC) proficient in homology directed repair (HDR) (Capecchi, 2005; Evans, 2001; Smithies, 2001). The application of this technique in other cell types and eukaryotic organisms has not been successful because of the inefficiencies of HDR mechanisms in most other organisms, including plants (Shukla *et al.*, 2009). Thus, one research focus has been to increase the efficiency of HDR. Several reports revealed that the generation of double-strand breaks (DSBs) stimulates the cellular repair process and increases the efficiency of HDR by several

orders of magnitude (Townsend *et al.*, 2009; Urnov *et al.*, 2005). Therefore, attempts have been made to use site-specific nucleases (SSNs) to generate site-specific DSBs and harness the cellular repair machinery to modify the sequence of the gene of interest (GOI) (Figure 1) (Durai *et al.*, 2005; Kim *et al.*, 1996). Generation of site-specific DSBs requires a protein that can be reprogrammed to bind to any sequence of interest and cleave double-stranded DNA simultaneously (Nakatsukasa *et al.*, 2005). Such proteins do not exist in nature but can be made as chimeric synthetic versions between a customizable or programmable DNA-binding module and a nonspecific nuclease catalytic domain.

The first customizable DNA-binding modules were generated from the common *Cys2-His2* zinc finger domain (Porteus and Carroll, 2005). Each zinc finger motif is composed of approximately 30 amino acids and binds to three nucleotides. Several approaches have been developed for the assembly of zinc finger arrays to user-defined target sequences including the modular assembly and oligomerized pool engineering (OPEN) (Maeder *et al.*, 2009). Assembled zinc finger arrays customized to bind to a user-selected DNA sequence were fused to the nonspecific catalytic domain of *FokI* endonuclease to create a chimeric zinc finger nuclease (ZFN) capable of generating site-specific DSBs (Morton *et al.*, 2006) (Figure 2a). These assembly approaches for user-selected DNA target sequences have proven to be laborious, resource intensive, time consuming, expensive and they suffer from high failure rates (Ramirez *et al.*, 2008). Because of these limitations, a major research goal has been to develop a DNA-binding module that can be easily engineered to bind to any sequence of interest with high efficiency and precision.

The phytopathogenic *Xanthomonas* bacteria secrete a group of transcription activator-like effectors (TALEs) into plant cells to naturally reprogramme the host transcriptional machinery to

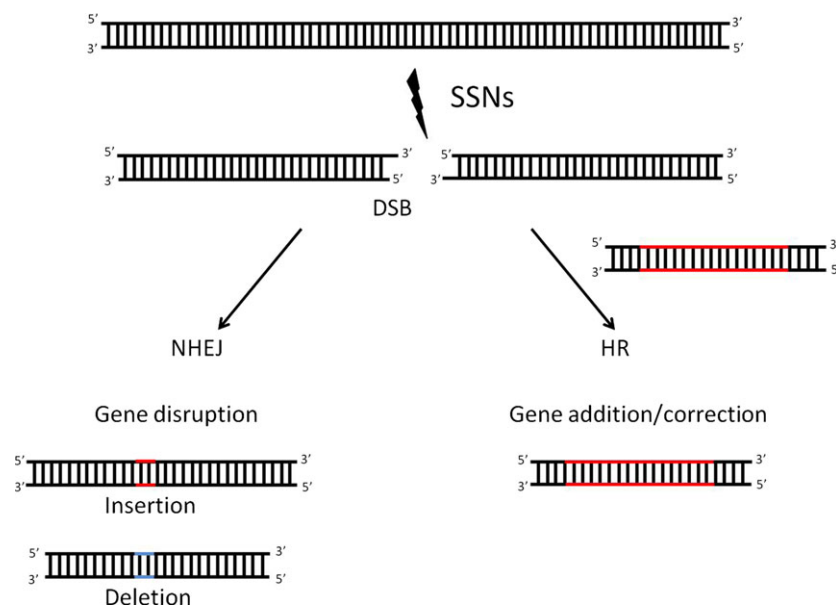


Figure 1 Genome editing mediated by site-specific nucleases (SSNs) mediate targeted genome editing across eukaryotic species. SSNs induce double-strand breaks (DSBs) that can be repaired by either the imprecise nonhomologous end joining (NHEJ) or precise homologous recombination DNA repair pathways. NHEJ rejoins the broken ends of DNA with random insertions or deletions of DNA nucleotides (indels). Homologous repair (HR), in the presence of a donor DNA sequence, can be harnessed to edit or replace the sequence of any given gene, resulting in gene correction or gene addition.

benefit the pathogen (Boch and Bonas, 2010). TALEs are secreted into plant cells and targeted to the plant cell nucleus wherein they bind to the promoter region of susceptibility or resistance genes to modulate the expression of these target genes (Romer *et al.*, 2009). Transcription activator-like effectors (TALE) proteins are by nature structurally similar to each other and contain three distinct domains including an N-terminal domain containing a type III secretion signal, a C-terminus domain containing a nuclear localization signal, an acidic activation domain (AAD) and a central DNA-binding domain (DBD) (Bogdanove and Voytas, 2011). The central DBD is composed of nearly identical 33–35 amino acids-long repeats with variable number among TALEs. The repeats possess hypervariable diresidues (RVDs) at positions 12 and 13 of each repeat. The RVDs dictate the repeat binding specificity to a single nucleotide in the DNA target sequence. The code of binding specificity was determined thusly: HD binds to C nucleotides, NI binds to A, NG binds to T and NN binds to A or G nucleotides (Boch *et al.*, 2009). More RVD specificities have recently been determined to include NK binding to G nucleotides (Morbiter *et al.*, 2010). Moreover, TALEs from the *Ralstonia solanacearum* phytopathogen have been characterized and their DNA binding specificities have also been determined (de Lange *et al.*, 2013; Li *et al.*, 2013b). Herein, the RVD ND binds specifically to C nucleotides, HN binds to A or G nucleotides, NH binds to G nucleotides and NP binds to all nucleotides. DNA-binding specificity can be customized by engineering the TALE repeats using RVD specificity, number and order. Several repeat assembly protocols have been devised to assemble TALE repeats in a user-defined order, number and sequence (Cermak *et al.*, 2011; Chen *et al.*, 2013; Li *et al.*, 2012a; Morbiter *et al.*, 2011; Reyon *et al.*, 2012; Schmid-Burgk *et al.*, 2013; Weber *et al.*, 2011). Moreover, TALE architecture that provides efficient binding and activity have been determined and used to generate TALENs for genome-editing purposes (Zhang *et al.*, 2011) (Figure 2b). Synthetic TALEs can be used in chimeric nucleases, transcriptional activators and repressors and have proven utility in both mammalian cells and plants (Cermak *et al.*, 2011; Hickey *et al.*, 2012; Liu *et al.*, 2014; Mahfouz *et al.*, 2011, 2012). TALEs have been used as a DNA-binding module fused to a variety of

functional domains for different genomic manipulations (Cong *et al.*, 2012; Geissler *et al.*, 2011; Li *et al.*, 2012a; Sanjana *et al.*, 2012).

Very recently, a new DNA-targeting platform has been developed for genome-editing applications. This genome-editing platform was developed from a natural adaptive immune system of bacteria and archaea called clustered regularly interspaced palindromic repeats (CRISPRs)/that includes the CRISPR-associated (Cas) type II nuclease system (Heidrich and Vogel, 2013). The CRISPR/Cas system is comprised of a Cas9 endonuclease protein and two small RNA molecules, namely CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) (Karvelis *et al.*, 2013). Recent studies have shown that the two small RNA molecules can be combined in a chimeric single guide RNA molecules (gRNA) (Hsu *et al.*, 2014) capable of directing the Cas9 endonuclease to the user-selected target sequence (Mali *et al.*, 2013) (Figure 2c). As the gRNA molecule mediates targeting information, easy and multiplexed editing across cell types and organisms is becoming increasingly possible. The facile engineering of the CRISPR/Cas system provides a cheap and rapid genome-editing platform that can be widely adopted by research laboratories for multiplexing genome-editing tasks.

Here, we review the discovery and development of the TALENs and CRISPR/Cas DNA-targeting platforms. We discuss the benefits and limitations of each system and also speculate about the opportunity for potential improvements and applications.

TALENs and mTALENs: protein-based DNA-targeting system

TALENs are TALEs fused to the cleavage domain of the *FokI* endonuclease for binding target DNA to create DSBs (Christian *et al.*, 2010; Mahfouz *et al.*, 2011; Miller *et al.*, 2011) (Figure 2b). In plants, DSBs are mainly repaired by the nonhomologous end joining (NHEJ) mechanism, which leads to small deletions or insertions (indels) (Figure 1). When donor DNA is introduced, sequence editing can be achieved (Zhang *et al.*, 2013) and these DSBs stimulate gene targeting efficiency by several orders of magnitude, which subsequently can facilitate potentially high

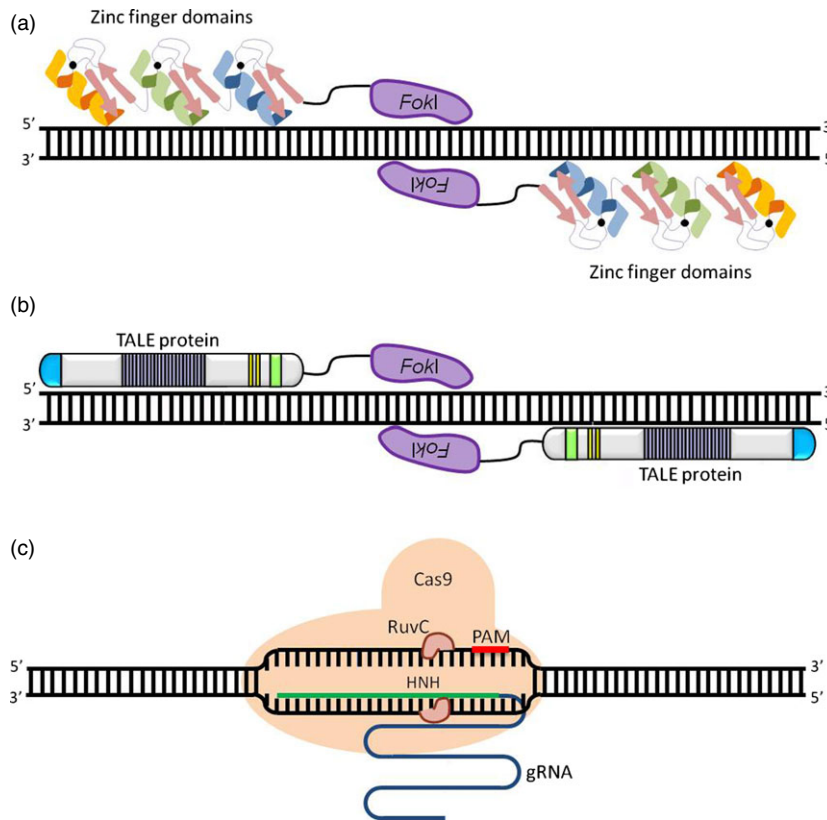


Figure 2 Schematic representation of various genome-editing platforms. (a) Zinc finger nucleases (ZFNs) are composed of DNA recognition domains and FokI nuclease catalytic domain fusions. Each zinc finger in the DNA recognition domains binds three nucleotides. On average three to four zinc fingers are fused to recognize 9–12 nucleotides. Two ZFNs are required to produce double-strand breaks (DSB) as the *FokI* domain requires dimerization to be catalytically active. (b) Transcription activator-like effector nucleases (TALENs) are composed of TAL central DNA-binding repeat domain and *FokI* catalytic domain fusions. DNA-binding specificity is determined by the 12th and 13th hypervariable residues of each repeat [repeat variable diresidue (RVD)]. Similarly, two TALENs heterodimer binding in a tail-to-tail orientation with proper spacer length to allow dimerization of the *FokI* domains are required for activity and DSB formation. (c) Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 mediates DSBs formation. Cas9 is guided to the 20 nt DNA target by a synthetic single guide RNA (gRNA) molecule composed of crRNA and tracrRNA. Cas9 recognizes a specific protospacer associated motif (PAM) sequence on the DNA (NGG—marked in red) and performs a complete cut with the two active nuclease domains RuvC and HNH.

efficiencies of gene stacking and replacement (Carroll, 2011) (Figure 1). TALENs have proven to be useful to generate site-specific DSBs in plant cells (Shan *et al.*, 2013; Zhang *et al.*, 2013). It should be noted that the ability to target TALENs to a specific genomic sequence permits multiple engineering possibilities including targeted mutagenesis and to add, delete or edit genes with precision.

For TALENs to function in genome editing, mostly to delete DNA, the *FokI* cleavage domain must dimerize to cleave both strands of the DNA target (Figure 2b). Therefore, two TALENs are used together to target the opposite DNA strands in a tail-to-tail orientation, with proper spacing between the two binding sites (Li *et al.*, 2011; Mahfouz *et al.*, 2011). The spacer length between homodimer and heterodimer binding is therefore dependent on the linker length, the sequence between the cleavage domain, as well as the repeat domain. Generally, longer linkers require longer spacers and vice versa. TALENs have been shown to be functional when spacer lengths range from 12–30 bp. The error-prone NHEJ or HDR pathways can potentially be utilized for different genetic modification outcomes. Among a wide variety of organisms, TALENs have proven to be efficient in generating genomic DSBs for the production of desired genetic modification (Bedell *et al.*,

2012; Carlson *et al.*, 2012; Cermak *et al.*, 2011; Li *et al.*, 2012b; Liu *et al.*, 2012), including yeast, plants, zebrafish and mammalian cells.

The requirement of *FokI* domain self-dimerization is a major impediment to efficiency and utilization for TALENs because it requires the co-expression of two separate TALE nucleases (TALEN) genes to form a functional heterodimer around the target sequence. Moreover, each monomer has the potential to create off-target events through perturbing the transcriptional machinery at nontarget sites, which might complicate the application of TALENs (Aouida *et al.*, 2014). Thus, an alternative to the *FokI* nuclease would be required to facilitate the generation of monomeric TAL-based nucleases (mTALENs). As only a single monomer is required for the activity, the off-target specificities could potentially be reduced by up to 50% when compared to TALENs. Monomeric TAL-based nucleases would be composed of a single polypeptide that is capable of generating a double- or single-strand breaks in the user-defined target sequence. There is a rich resource of nuclease catalytic domains from microbial or other eukaryotic sources, including humans, which can be tested for the generation of such monomeric TAL-based nuclease. For example, I-TevI is an isoschizomer of a GIY-YIG homing endonu-

lease family, with the recognition site for cleavage of CNNNG, which has the critical G that is optimally positioned ~28 bp from where the helix-turn-helix module of the *I-TevI* DBD interacts with substrate DNA. The linker, situated in between the catalytic domain and the DBD, is a complex structure, consisting of defined structural elements with distinct roles in I-TevI function (Bujnicki *et al.*, 2001; Edgell and Shub, 2001; Van Roey *et al.*, 2002). The primary role of the linker is to position the nuclease domain on the DNA substrate for cleavage at the CNNNG motif, located at a specified distance (10 bp) from the binding site on the naturally occurring *I-TevI* substrates (Beurdeley *et al.*, 2013; Dean *et al.*, 2002; Kleinstiver *et al.*, 2012). The unique biochemical properties of the I-TevI nuclease might represent a viable alternative to the *FokI* nuclease for genome-editing applications. As the *I-TevI* nuclease domain was shown to be portable to ZF DBDs, it may be applicable to TALEs too (Kleinstiver *et al.*, 2012). It is expected that the mTALENs-TevI, that is, mTALENs will be able to cleave DNA in a site-specific fashion and generate DSBs and thereby stimulating DNA recombination to provide a useful tool for targeted modification of the genome (Beurdeley *et al.*, 2013).

mTALENs could provide multiple benefits. First, the use of single protein to create DSBs could lead to simple and facile application of genome editing across eukaryotes with half the vector design and cloning effort. Second, enhanced cleavage specificity will significantly lower the potential off-target events. However, the caveat to this statement is that the sequence targeted by the TALEN would be halved. Thus, research around this topic would be important to consider in mTALEN development. Third, there might be wider applications with regards to targets, cell types and organisms.

To minimize the potential deleterious side effects of TALENs, it will be highly desirable to use TALE nickases to introduce site-specific nicks in the genome to initiate a DNA repair that results in HDR to increase the scope and applicability of nondeletion genome-editing applications. For example, by fusing TALEs to the nickase domain of HNH endonucleases (HNHE) to form an mTALENkase could open the door to the production of a new class of mTALENs. HNHEs have been shown to be site-specific and strand-specific nicking endonucleases with 3–7-bp specificities and a minimal nicking domain of 76 amino acid residues was identified from *Bacillus* phage c (Xu and Gupta, 2012). These next-generation mTALENs, mTALENs and mTALENkases would greatly enhance and widen the genome-editing tool kit that would be especially valuable, but not restricted, for plant biotechnology innovations.

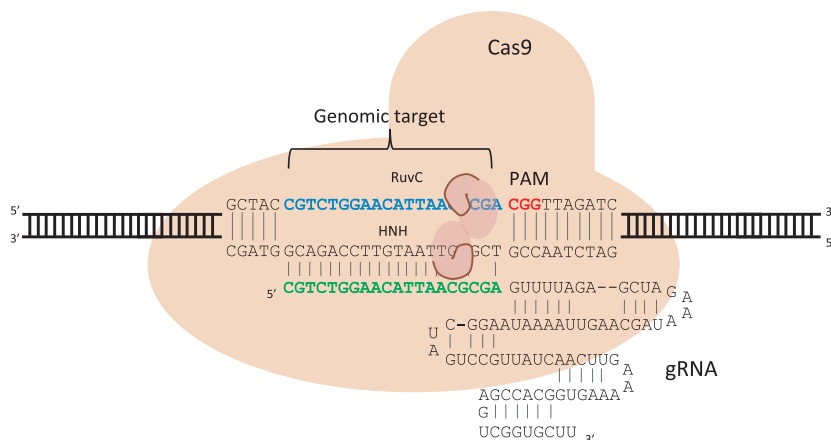
CRISPR/Cas system: an RNA-based DNA-targeting module

The most recent addition to the genome-engineering toolbox is the clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated system (Barrangou, 2012). The CRISPR/Cas system is an adaptive immune system that helps protect bacteria and archaea from phages or conjugative plasmids through horizontal gene transfer that could be potential invaders (Barrangou and Marraffini, 2014). The type II CRISPR/Cas9 system is composed of Cas9 endonuclease and two RNA molecules, namely crRNA and tracrRNA. The crRNA and tracrRNA require RNaseIII enzyme for processing and maturation of the crRNA molecule, which guides the Cas9 endonuclease to its DNA target (Gasiunas *et al.*, 2012; Jinek *et al.*, 2012). Very recently, this system has been developed as a genome-engineering platform across eukaryotic species. It has been shown that the two RNA molecules can be combined as a single guide RNA molecule (gRNA) capable of directing Cas9 protein to its target (Cong *et al.*, 2013; Mali *et al.*, 2013) (Figure 3). As the small gRNA molecule carries the target specificity, it has become quite simple and easy to engineer single and multiple target specificities. Such unprecedented facile engineering has made the CRISPR/Cas9 the platform of choice for genome engineering, even though it is a very new system in biotechnology (Sander and Joung, 2014). There is a surge in the application of this technology for targeted genome editing across eukaryotic species (Sander and Joung, 2014). However, apparently the ease of engineering is accompanied by a pronounced high rate of off-target binding. Several reports have indicated an increased off-target activities of the enzyme even in the once thought most conserved seed sequence (Fu *et al.*, 2013; Liu *et al.*, 2014). However, it may be possible to generate CRISPR/Cas9 derivatives with both high efficiency and specificity. It is also expected that a novel class of DNA or RNA-guided nucleases based on the prokaryotic argonaute proteins could be generated in the near future to address some of the limitations of the current editing platforms (Sheng *et al.*, 2014; Swarts *et al.*, 2014).

The specificity of CRISPR/Cas9: a problem with multiple possible solutions

There have been several recent reports noting off-target host effects of CRISPR/Cas9 genome editing. Mismatches in the target sequence are tolerated by the system (Cradick *et al.*, 2013). It is

Figure 3 Targeted genome editing via RNA-guided Cas9 endonuclease. Cas9 endonuclease is guided to the target DNA sequence called protospacer (shown in blue) by a single guide RNA (gRNA) molecule that contains a 20 nt spacer sequence (shown in green). Protospacer associated motif (PAM) sequence (shown in red) is a prerequisite for binding and cleavage and is situated upstream of the protospacer. The Cas9 two nuclease domains RuvC and HNH, each cleave one strand of a double-stranded DNA molecule (dark pink).



quite likely that the mismatch tolerance is inherently found in the natural system to allow for the recognition of rapidly evolving viral genomes (Barrangou and Marraffini, 2014). Thus, having such ability to tolerate mismatches provide bacteria and archaea with wide-spectrum defence against invading viral and conjugative plasmid DNA. Target sequences carrying double, triple or quadruple mismatches were well tolerated with mutation frequencies equal to that of the wild-type sequence (Hsu *et al.*, 2013). Generally, mismatches distal from the protospacer associated motif (PAM) sequence are well tolerated (Liu *et al.*, 2014). Mismatches proximal to the PAM sequences can also produce off-target issues (Fu *et al.*, 2013; Hsu *et al.*, 2013). There have been two recent publications on the target specificity of a catalytically inactive Cas9 (dCas9) variant and the native Cas9, wherein the extent of off-target effects were investigated using chromatin immune precipitation and high-throughput sequencing (ChIP-Seq) (Kuscu *et al.*, 2014; Wu *et al.*, 2014). The dCas9 exhibited elevated off-target binding, mostly in open chromatin and that the PAM-proximal region (seed sequence) played a major role in determining the binding specificity. Moreover, the native Cas9 cleaved some but not all predicted off-target binding sites. These findings prompts the need to innovate Cas endonuclease variants for altered PAM sequence requirements for improved specificity (Sander and Joung, 2014). The on- and off-target cleavage activities seem to vary among cell types, and it is quite possible that there would be variation across eukaryotic species in this regards. Studies should focus on delineating clear rules to the design of gRNA for target specificity. Current studies are focused on developing Cas9 variants or strategies to improve the overall efficiency and specificity of the system (Ran *et al.*, 2013). In one example, two Cas9 nickases guided by two gRNA targeting the sense and antisense strands were used to generate DSB (Cradick *et al.*, 2013; Ran *et al.*, 2013). Even though this strategy improved the overall specificity of the system, it also generated genome nicks, which can induce mutagenesis. This is a major concern to genetic medicine applications (Cradick *et al.*, 2013; Sander and Joung, 2014), but this issue might not be as crucial for plant biotechnology, however, regulators of biotechnology might judge otherwise, which would motivate the tweaking of a CRISPR

system to render high fidelity, precision, and environmental robustness. For example, dCas9: FokI fusions have been reported to significantly reduce the off-target activities (Guilinger *et al.*, 2014; Tsai *et al.*, 2014).

Characteristics of an ideal site-specific synthetic nuclease for plant genome editing. Which is best: ZFNs, TALENs or CRISPR/Cas9?

As synthetic nucleases are used to manipulate complex genomes, they need to be highly specific with minimal or no off-target activities to avoid unintended modifications and cellular toxicity. Further desirable features of a genome-editing system include facile engineering of any user-defined binding specificity and efficient delivery into target cells. Compared to ZFNs, TALENs are easier to design and engineer, but they suffer from off-target binding (Mahfouz and Li, 2011). CRISPRs appear to be the easiest of all to design and implement, as only one nuclease is needed and the gRNA component is facile to construct and engineer into cells. Targeted genome modification has been accomplished using all three systems. ZFN and TALEN specificity/binding depends on protein-DNA interactions that might have repeat context dependence and affected by the epigenetic status (Li *et al.*, 2013b). In contrast, the CRISPR/Cas9 system depends on Watson–Crick base pairing, which is highly predictable and is the current favourite system for molecular biologists to rapidly edit genomes. Several reports have indicated that the CRISPR/Cas system has modified gene targets that were recalcitrant to TALENs (Cong *et al.*, 2013; Ran *et al.*, 2013). One drawback of CRISPRs/CAS9 is that it suffers from elevated rates of off-target activities. However, off-target effects appear to vary among cell types and organisms. The question of which platform to use will largely depend on the specificity of generating the intended modification, reproducibility, efficient delivery to various cell types and organisms, facile engineering and customizability, affordability, versatility and potential improvements (Table 1).

Intellectual property (IP) will also play a role in private sector research and development. In general, the newer the system, the less is known about dominating patents and other IP issues

Table 1 A systematic comparison between TALE nucleases and clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 genome-engineering systems

	TALENs	CRISPR/Cas9
Construction	Protein engineering of the repeat variable diresidue sequence, number and order of the repeats in the DNA binding domain	20 nucleotide sequence in small single guide RNA (gRNA) molecule
Delivery	Overexpression of two TALENs to form a heterodimer around the target site	Overexpression of the gRNA molecule in Cas9 overexpression background
Mechanism of action	Introduction of double-strand breaks (DSBs) in target DNA	Introduction of DSBs in target DNA by wtCas9 or single strand nicks by Cas9 nickase
Cleavage efficiency	Efficient	Highly efficient
Off-target effects	Limited off-target activities, not fully studied in plants	No off-targeted activities reported in plants, but high off-target levels reported in other systems. Further studies are needed
Feasibility of library construction and transformation	Technically challenging in generation and application in plants	Highly feasible in Cas9 overexpression background
Portability to viral delivery	Not portable due to size constraints	gRNA molecules portable to RNA viruses
Affordability	Affordable but resource intensive	Highly affordable
Applicability	All transformable plant species	All transformable plant species

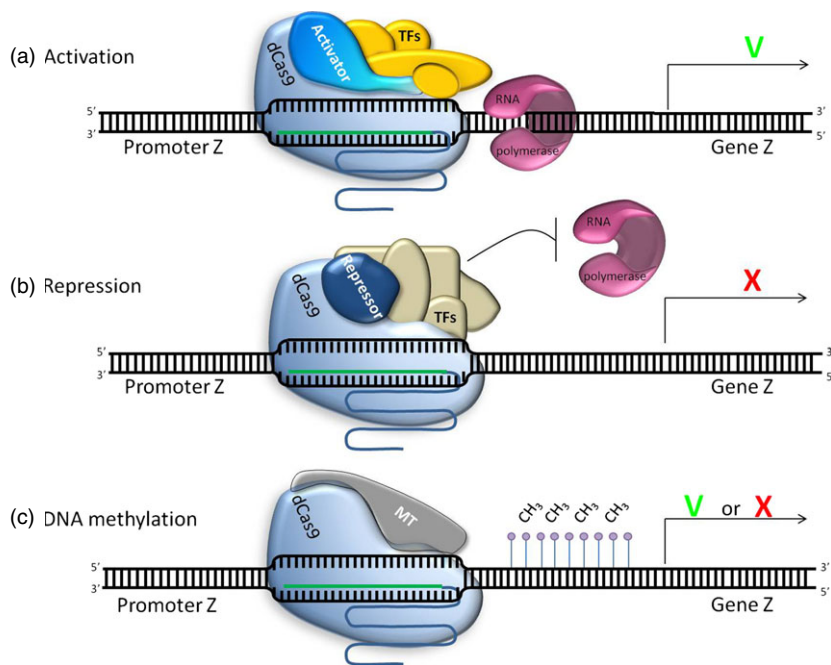


Figure 4 Targeted genome regulation mediated by dCas9-based synthetic transcription factors. (a) dCas9—activator fusions potentially enhance gene expression by recruitment of transcription factors and RNA polymerase II. (b) Likewise, dCas9 fusions with potent repressors domains could either prevent the RNA polymerase from binding to the promoter region of specific gene or abrogating the elongation process resulting in gene repression activities. (c) dCas9 synthetic protein fusions with methylases/demethylases or chromatin modifiers could regulate the transcription of the targeted gene.

(Webber, 2014). Nonetheless, CRISPR/Cas seems to hold the most potential in broad usability for genome editing (Fauser *et al.*, 2014; Feng *et al.*, 2014; Li *et al.*, 2013a).

Of all the potential problems, off-targeting activities pose serious challenges to all systems, especially when it comes to genomic medicine applications where specificity is critical. The

presence of Cas9 variants with different protospacer associated motif (PAM) requirements suggests the possibility of improving the specificity of the CRISPR/Cas system (Chylinski *et al.*, 2014; Kim and Kim, 2014). We can expect further improvements of the CRISPR/Cas system will be achieved through protein engineering and directed evolution (Guilinger *et al.*, 2014; Jinek *et al.*, 2014;

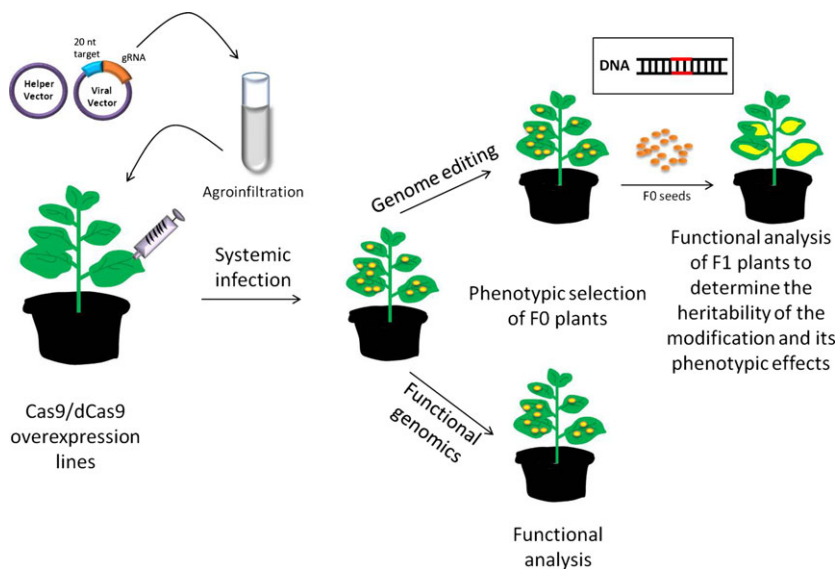


Figure 5 Viral-mediated delivery of clustered regularly interspaced palindromic repeats (CRISPR) single guide RNA (gRNA) molecules for targeted genome editing and regulation. A viral vector harbouring a gRNA backbone and 20 nucleotide target sequence, and a helper vector are transformed into *Agrobacterium tumefaciens* and co-infiltrated into an overexpression plant line of either Cas9 or dCas9. In this example, the helper vector facilitates the movement of the virus from one cell to another mediating the expression of the gRNA molecules in each individual cell. Because the systemic infection is fast and effective, the F₀ generation of plants can be assayed for the modification in the leaves that are systemically infected. Depending on the application, plants can be analysed, for example, for the genome changes through detection of DNA indels at specific locus via Surveyor, other sequence-based assays or using gene function analysis by monitoring the levels of gene expression linked to the expected phenotype. For genome-editing applications, this virus-mediated system can be used because the mutation can be passed to next generation because of proliferation of the virus in plant meristematic cells. The efficiency and precision of genome modification can be assayed in the F₁ generation to determine the heritability of the modification and its functional consequences.

Nishimasu *et al.*, 2014; Tsai *et al.*, 2014). Several studies have reported no off-target activities in plant cells making the CRISPR/Cas9 the system of choice for plant genome engineering (Feng *et al.*, 2014; Nekrasov *et al.*, 2013; Zhang *et al.*, 2014). However, further comparative studies on the binding specificities and activities of CRISPR/Cas9 and TALENs are needed in various different plant species.

Targeted genome editing and regulation for plant biotechnological applications

One of the major challenges in plant biotechnology is the ability to regulate the transcription of endogenous genes (Figure 4). Fusion proteins that consist of dCas9 (nuclease-dead Cas9) and repressor domains (Figure 4b) or activator domains (Figure 4a) hold great promise for controlling targeted gene expression in space and time. Such systems could be applied for the regulation of plant metabolic pathways and, therefore, for crop improvement. Moreover, the ability to modulate gene expression will be useful for interrogating gene functions, gene networks and, therefore, accelerating the development of synthetic biology applications. RNA-guided transcriptional regulation will become an easy and feasible platform for the regulated expression of endogenous genes.

As a gRNA molecule mediates the Cas9 targeting in CRISPRs, it would be possible to express gRNA libraries in plants overexpressing the Cas9 endonuclease and identify novel traits through different selection schemes. Cloning and sequencing of the gRNA molecule can identify specific genes related to the recovered phenotypes. Candidate genes can be identified and sequenced to map the mutations. This approach would provide a discovery platform that links a phenotype to genotype and help identify novel traits of agricultural importance. Moreover, gRNA libraries can be delivered and transformed transiently *via* virus-based system in a heterozygous overexpression line of Cas9, and it is possible to recover plants carrying only the desired mutations and lacking any CRISPR/Cas9 system components (Figure 5) (Marton *et al.*, 2010). Furthermore, a CRISPR/Cas9 genome regulation platform can be built by generating an overexpression line of catalytically inactive dCas9. Viral-mediated transient expression of a gRNA library, which might be gene-family specific, will provide an effective means for the genome-wide interrogation of the genome for functional analysis. These CRISPR/Cas9-based genome editing and genome regulation platforms can be applied to many plant species for functional analysis and trait discovery and development.

Perspectives

The current genetic engineering paradigm relies on inserting exogenous DNA into a plant genome. Opposition to plant genetic engineering persists, even though the weight of evidence indicates transformation, *per se*, is benign to human health and the environment. Many governments have significant regulatory hurdles that impede the development and commercialization of transgenic crops. The decision of whether to regulate or not most often hinges on the basis of engineering methods rather than phenotype or real risks; the consequence of this fact is, undoubtedly, less innovation and humanitarian benefit. New genome-editing tools have the potential to increase the pace of genome modification, but the possible change of regulatory landscape is unknown. Next-generation

mTALENs and improved versions of the CRISPR/Cas9 system represent a major advance in the techniques used to improve crop plants. These new technologies feature enhanced precision compared with many unregulated plant breeding technologies, such as those that rely on random mutagenesis (Liu *et al.*, 2013). The continued goal for these new genome-editing technologies will be to increase precision of changes while minimizing off-target effects to expand the range of traits in agriculture (Mahfouz and Li, 2011).

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