TALENs: Customizable Molecular DNA Scissors for Genome Engineering of Plants

Article *in* Journal of Genetics and Genomics · June 2013

DOI: 10.1016/j.jgg.2013.03.009 · Source: PubMed

2 authors:

Kunling Chen
Chinese Academy of Sciences
40 PUBLICATIONS **4,980** CITATIONS
SEE PROFILE

Caixia Gao
Institute of Genetics and Developmental Biology, CAS
119 PUBLICATIONS **10,564** CITATIONS
SEE PROFILE

Available online at www.sciencedirect.com
SciVerse ScienceDirect
Journal of Genetics and Genomics 40 (2013) 271−279
JGG

REVIEW

TALENs: Customizable Molecular DNA Scissors for Genome Engineering of Plants

Kunling Chen, Caixia Gao*

State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

Received 18 February 2013; revised 18 March 2013; accepted 18 March 2013
Available online 26 March 2013

ABSTRACT

Precise genome modification with engineered nucleases is a powerful tool for studying basic biology and applied biotechnology. Transcription activator-like effector nucleases (TALENs), consisting of an engineered specific (TALE) DNA binding domain and a *Fok* I cleavage domain, are newly developed versatile reagents for genome engineering in different organisms. Because of the simplicity of the DNA recognition code and their modular assembly, TALENs can act as customizable molecular DNA scissors inducing double-strand breaks (DSBs) at given genomic location. Thus, they provide a valuable approach to targeted genome modifications such as mutations, insertions, replacements or chromosome rearrangements. In this article, we review the development of TALENs, and summarize the principles and tools for TALEN-mediated gene targeting in plant cells, as well as current and potential strategies for use in plant research and crop improvement.

KEYWORDS: TALENs; Genome engineering; Targeted gene modification; Plant

INTRODUCTION

Targeted genome modification (TGM), mediated by engineered nucleases, has been widely used to investigate gene function and expand biotechnology applications in yeast (Scherer and Davis, 1979), fruit fly (Bibikova et al., 2002), mice (Capecchi, 2005), human cell lines (Urnov et al., 2005), plants (Wright et al., 2005; de Pater et al., 2009) and many other organisms (Remy et al., 2010; Urnov et al., 2010). Reliable and efficient methods for obtaining site-specific modifications in plants are long sought-after goals for basic plant research and crop improvement (Pennisi, 2010). Although first demonstrated in the late 1980's, TGM in plants was far from routine because of its extremely low efficiency (typically about 10^{-5}) (Paszkowski et al., 1988; Halfter et al., 1992). The critical step in TGM is the introduction of DNA double-strand breaks (DSBs) at given genomic sites. It was soon discovered that engineered nucleases could generate DSBs and consequently activate DNA repair to seal the breaks along with any modifications such as mutations, insertions, replacements, and chromosomal rearrangements (Rouet et al., 1994).

Traditionally, TGM was mostly performed using zinc finger nucleases (ZFNs), artificial nucleases that consist of a synthetic ZFN domain fused to a *Fok* I cleavage domain (Urnov et al., 2010; Carroll, 2011). ZFNs have been used to modify endogenous genes in a wide range of organisms and cell types, and plant species including *Arabidopsis* (Osakabe et al., 2010; Zhang et al., 2010), tobacco (Townsend et al., 2009), maize (Shukla et al., 2009) and soybean (Curtin et al., 2011). Major constraints on ZFN application include the limited number of available target sites, more context dependence effects between the repeat units, low targeting efficiency and specificity, and

* Corresponding author. Tel/fax: +86 10 6480 7727.
E-mail address: cxgao@genetics.ac.cn (C. Gao).

http://dx.doi.org/10.1016/j.jgg.2013.03.009

frequent off-target effects caused partly by nonspecific DNA binding (DeFrancesco, 2011).

More recently, transcription activator-like effector nucleases (TALENs) have emerged as alternatives to ZFNs for TGM, and have been shown to have great potential for precise genome manipulation (Christian et al., 2010). Like ZFNs, TALENs consist of an engineered specific TALE DNA binding domain and a *Fok* I cleavage domain. The customizable TALE (Transcription activator-like effectors) DNA binding domain, composed of several nearly identical tandem repeat arrays, can target any given sequence according to a simple repeat variable di-residue (RVD)-nucleotide recognition code (Bogdanove et al., 2010; Bogdanove and Voytas, 2011). Within the past three years TALEN-mediated genome modification has been widely adopted in yeast (Li et al., 2011), nematode (Wood, 2011), fruit fly (Liu et al., 2012), rat (Tesson, 2011; Tong et al., 2012), human somatic and pluripotent cells (Hockemeyer et al., 2011; Miller et al., 2011), silkworm (Ma et al., 2012), livestock (Carlson et al., 2012), plants (Li et al., 2012b; Mahfouz et al., 2011; Shan et al., 2013; Zhang et al., 2013), *Xenopus* embryo (Lei, 2012), zebrafish (Huang et al., 2011; Sander et al., 2011; Bedell, 2012; Cade et al., 2012; Dahlem et al., 2012; Moore, 2012) and many other organisms, and is recognized as a major breakthrough in the core technology of genome engineering. Genome editing with engineered nucleases was named 2011 "Method of Year" (2011), and one year later genome engineering using TALENs and Cas9 was crowned 2012 "Breakthrough of the Year" by Science (2012).

Here we review the DNA targeting characteristics of TALENs and describe the available TALEN construction platforms. We also summarize the applications that have been made in plants, and discuss future work to enhance the utility of these tools in plant research and crop improvement.

DNA BINDING SPECIFICITY

TALEs are major virulence factors secreted by the plant pathogenic bacterial genus *Xanthomonas*, which causes disease in plants such as rice and cotton (Boch and Bonas, 2010; Bogdanove et al., 2010). TALEs are injected into host cells through the type III secretion system and interfere with cellular activities by activating the transcription of specific target genes (Bogdanove et al., 2010). They have specific structural features, including secretion and translocation signals in the N-terminal region, nuclear localization signals (NLS) and an acidic transcription-activation domain (AD) in the C-terminal region and a central DNA binding domain (DBD) with 33−35 nearly identical long amino acid repeats, followed by the last module which contains only 20 amino acids (refers to as "half repeat") (Fig. 1A). The repeat variable di-residue (RVD) at positions 12 and 13 of each repeat dictates the specificity of repeat binding to a nucleotide in the DNA target (Fig. 1A). This RVD-nucleotide preference was first identified as the DNA sequence recognition code of TALEs in 2009 by two independent groups (Boch et al., 2009; Moscou and Bogdanove, 2009). According to this code, the HD repeat specifies C, NG specifies T, NI specifies A, NN specifies G or A, N* specifies C, IG specifies T, and NS specifies A, C, G, or T. Although many natural RVDs have been discovered, four of them, HD, NN, NI, and NG, account for 75% of the total (Moscou and Bogdanove, 2009) (Fig. 1A).

The DNA binding specificity of a TALE is determined by its repeat number and the sequence of the RVD: the repeat number determines the length of the target sequence, while the RVD corresponds directly to the nucleotide in the target site. Moreover, recognition sites are always preceded by a thymine (T) before the first repeat in the array and this is the only critical rule for TALE targeting (Boch et al., 2009).

The three-dimensional structures of TALE−DNA complexes, reported in two back-to-back papers in 2012 (Deng et al., 2012a; Mak et al., 2012), provided detailed additional information supporting the one-to-one RVD-nucleotide recognition code. It was shown that each TAL repeat comprises two helices connected by a short RVD-containing loop, and all repeats form a right-handed superhelical structure that tracks along the sense strand of the DNA duplex, with the RVDs contacting the major groove. The two hypervariable residues in the RVD loops have different biochemical roles. The second amino acid of the RVD (position 13) mediates specific recognition of the sense strand DNA base, whereas the first amino acid (position 12) does not directly contact the DNA but instead helps to stabilize the repeat structure.

From a target DNA sequence, researchers can in principle analyze the number and order of the required RVDs and design a TALE protein to target a given site in any organism. The simplicity and the modularity of the repeats enable rapid construction of TALE proteins fused to a number of functional domains to achieve site-specific modifications of the genome. These functional domains include repressors, activators, nucleases, nickases, recombinases, methylases, integrases and others (Fig. 1B).

Thus, using HD = C, NN = G, NI = A, and NG = T as code, customizable TALENs have been created and widely used in TALEN-mediated TGM. In addition, since the HD and NN are regarded as strong RVDs, while NI, NG or NK are weak ones, Streubel et al. (2012) suggested that the former should be employed and stretches ($\geq$6) of weak RVDs should be avoided in the design of efficient TALEs. The uses of some rarely used RVDs have also been studied. For example, NK and NH were found to be more specific for G than NN but have lower affinity (Moscou and Bogdanove, 2009; Miller et al., 2011; Zhang et al., 2011; Streubel et al., 2012), while N* or NG were shown to bind 5mC and so overcome methylation of the cytosine (Deng et al., 2012b; Valton et al., 2012).

TALEN ASSEMBLY PLATFORMS

Because of the long and highly repetitive nature of the DBDs of TALEs, it is a major challenge to construct engineered TALENs by ordinary PCR and traditional cloning techniques. Although custom-engineered TALEs have become available commercially through Cellectis Bioresearch and Life

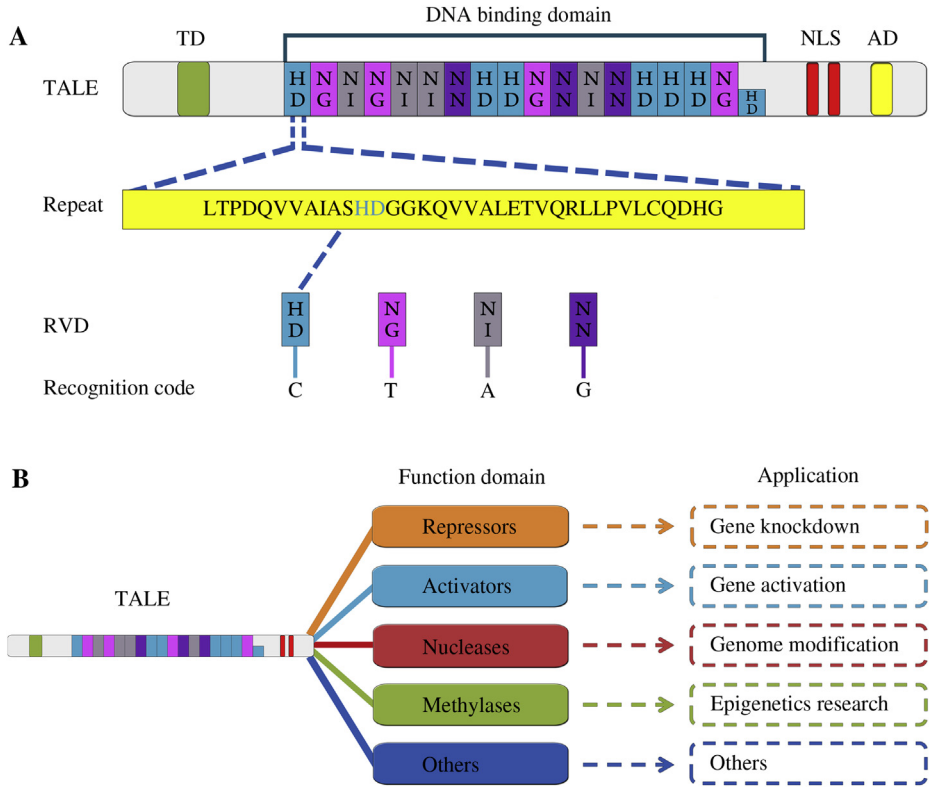

Fig. 1. Overview of TALE structure, DNA recognition code and TALE-based designer proteins.
A: TALE structure and DNA recognition code: a typical TALE structure (top) comprises an N-terminal translocation domain (TD), a central DNA binding domain (DBD), two nuclear localization signals (NLS) and a transcriptional activation domain (AD) in the C-terminal region. The DBD is composed of several tandem repeats which end with a half repeat. Each repeat consists of 34 nearly identical amino acids except for the central repeat variable di-residue (RVD) in positions 12 and 13. The one-to-one RVD-nucleotide code of the four common RVDs used for DNA specific targeting in TALENs (TALE nuclease) is shown below. **B:** TALE proteins: an optimal TALE scaffold including the central DBD can be fused to some functional domain, such as repressor, activator, nuclease or methylase, to generate TALE-based designer proteins for site-specific modification of the genome.

Technologies, they are too expensive to adopt routinely like DNA sequencing. In the past three years, with more understanding of the features of TALENs, a variety of rapid TALEN assembly methods have been invented by a number of molecular genetics laboratories. Depending on their assembly theories, all can be divided into three main platforms, which use (1) standard cloning assembly methods, (2) Golden Gate assembly methods and (3) solid-phase assembly methods, respectively (Joung and Sander, 2012; Xiao et al., 2013).

The standard cloning assembly methods (also known as sequential assembly) use standard restriction digestion and ligation reactions to create a final assembled array in a parallel hierarchical process. Three methods, namely unit assembly (Huang et al., 2011), REAL (Sander et al., 2011) and REAL-Fast Assembly (Reyon et al., 2012a), respectively, have been successfully developed and applied in different organisms.

Of the three platforms, Golden Gate assembly technology has been widely used, with the advantage of being simple, fast and inexpensive (Cermak et al., 2011; Li et al., 2011; Sander et al., 2011; Weber et al., 2011; Zhang 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. For example, a custom TALEN with 15−31 repeats can be efficiently designed and made by two-step ligation using the Golden Gate assembly protocol invented by the Voytas laboratory (Cermak et al., 2011). The Golden Gate assembly methods depend on sequencing to identify the correct clone, and it usually requires 3−5 days to accomplish assembly. Up to now, most TALENs targeting plant genes have been constructed by the Golden Gate method (summarized in Table 1).

Four high-throughput TALEN assembly methods, FLASH (Fast Ligation-based Automatable Solid-phase High-throughput), ICA (Iterative Capped Assembly), Chip and LIC (Ligation Independent Cloning), dependent on solid-phase synthesis, have been described. FLASH enables assembly of fragments encoding 96 arrays in less than a day (Reyon et al., 2012b). ICA is a protocol for performing iterative ligation on solid-phase magnetic beads, developed by the Church laboratory (Briggs et al., 2012). DNA repeat monomers are added individually to a growing chain and hairpin “capping” oligonucleotides are used to block incompletely extended chains. Using ICA, the synthesis of TALEs up to 21 monomers in length and their ligation into a nuclease-carrying backbone vector can be accomplished within 3h (Briggs et al., 2012). This method can greatly increase the frequency of the full-length

Table 1
Examples of targeted gene modification using TALENs in plants

Plant species	Gene name	Modification type	TALEN construction platform	Mutation	Reference
Arabidopsis	*ADH1*	NHEJ	Golden Gate	Gene knockout	Cermak et al., 2011
Tobacco	EBE of *Hax3*	NHEJ	Not available	Gene knockout	Mahfouz et al., 2011
Tobacco	*SurA*, *SurB*	NHEJ, HR	Golden Gate	Gene knockout, insertion and replacement	Zhang et al., 2013
Rice	EBE of *AvrXa7* and *PthXo3*	NHEJ	Golden Gate	Gene knockout	Li et al., 2012b
Rice	*OsDEP1*	NHEJ	Golden Gate	Gene knockout, large deletion, inversion	Shan et al., 2013
Rice	*OsBADH2*	NHEJ	Golden Gate	Gene knockout	Shan et al., 2013
Rice	*OsCKX2*	NHEJ	Golden Gate	Gene knockout	Shan et al., 2013
Rice	*OsSD1*	NHEJ	Golden Gate	Gene knockout	Shan et al., 2013
Brachypodium	*BdABA1*	NHEJ	Golden Gate	Gene knockout	Shan et al., 2013
Brachypodium	*BdCKX2*	NHEJ	Golden Gate	Gene knockout	Shan et al., 2013
Brachypodium	*BdSMC6*	NHEJ	Golden Gate	Gene knockout	Shan et al., 2013
Brachypodium	*BdSPL*	NHEJ	Golden Gate	Gene knockout	Shan et al., 2013
Brachypodium	*BdSBP*	NHEJ	Golden Gate	Gene knockout	Shan et al., 2013
Brachypodium	*BdCOI1*	NHEJ	Golden Gate	Gene knockout	Shan et al., 2013
Brachypodium	*BdRHT*	NHEJ	Golden Gate	Gene knockout	Shan et al., 2013
Brachypodium	*BdHTA1*	NHEJ	Golden Gate	Gene knockout	Shan et al., 2013

final product. Chip is another magnetic bead-based TALE assembly method, which allows the synthesis of over one hundred TALEs of 16−20 repeats in three days (Wang, 2012). LIC is based on a library of DNA constructs encoding individual TALE repeat unit combinations that are designed to anneal with the overhangs of other fragments in a highly specific manner without prior ligation. Researchers can assemble more than 600 TALENs with exceptional fidelity in a single day (Schmid-Burgk et al., 2012). These high-throughput TALEN assembly systems make it possible to modify genes on a genome-wide scale.

Researchers can obtain reagent kits for the above platforms from the non-profit plasmid distribution service Addgene (http://www.addgene.org/talen/). And, more conveniently, sources of TALENs including target design and prediction, assembly methods, reference collections, protocols and materials, and even newsgroups for discussion, are easily found on websites such as TALEN-NT (Doyle et al., 2012), idTALE (Li et al., 2012a) and EENdb (Xiao et al., 2013). Thus very straightforward working methods for generating TALENs are being developed.

TYPES OF GENOME MODIFICATION

Since *Fok* I functions as a dimer, TALENs designed as pairs allow two monomers to bind at two adjacent sites separated by a DNA spacer that allows *Fok* I to form dimers to cleave the given target sites. Consequently, DSBs induced by TALENs will activate DNA repair either by non-homologous end joining (NHEJ) or homologous recombination (HR). If DSBs are repaired by NHEJ, an error-prone pathway which often simply rejoins broken DNAs imprecisely, this will lead to frame-shift mutations with small insertions or deletions (indels) at or near the DSB, so this repair pathway can be used to create knock-out mutants. Alternatively, if DSBs are repaired by HR, which is stimulated by the homologous DNA template surrounding the DSBs, HR can be employed to create site-specific sequence modifications or insertions. Through NHEJ or HR, TALENs can be used to perform TGMs including mutations, insertions, replacements and chromosome rearrangements (Fig. 2).

Targeted gene mutagenesis

Targeted gene mutagenesis, the most common and simplest means of TGM, disrupts gene function by taking advantage of errors introduced by NHEJ. To disrupt a gene, TALENs should be designed to target the open reading frame (ORF) of a gene, particularly in the exons in the first 1/3 to 1/2 of an ORF. Indels induced by NHEJ can be introduced subsequently at predetermined sites to cause frame-shift mutations and knockout gene function. Gene mutations, induced in several plant species, such as *Arabidopsis* (Cermak et al., 2011), *Brachypodium* (Shan et al., 2013), rice (Shan et al., 2013), tobacco (Zhang et al., 2013), and many other organisms (Joung and Sander, 2012; Streubel et al., 2012; Tong et al., 2012), are the most important types of TGMs mediated by TALENs that have been reported to date.

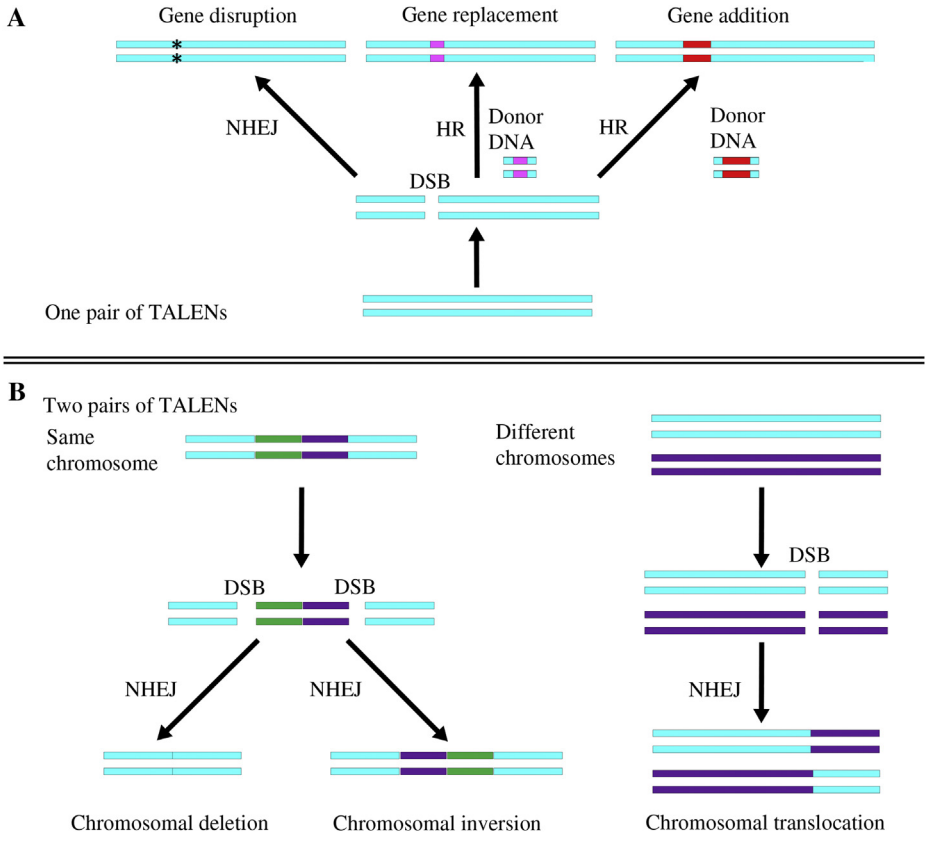

Fig. 2. Genome modification with TALENs.

A: genome modification with one pair of TALENs. Double-strand breaks (DSBs) induced by one pair of TALENs will activate DNA repair mechanisms either by non-homologous end joining (NHEJ) or homologous recombination (HR). Through NHEJ, DSBs lead to frame-shift mutations with small insertions or deletions (indels) and result in gene disruption (left panel); HR, stimulated by the homologous DNA template, leads to gene replacement (middle panel) or gene insertion (right panel). **B:** genome modification with two pairs of TALENs. The introduction of two pairs of TALENs targeted at two sites on the same chromosome can cause large deletions or inversions (left panel). The introduction of two nuclease-induced DSBs on different chromosomes can lead to translocations or multiple gene disruptions (right panel).

Targeted gene insertion

Traditional transgene methods typically transform and integrate foreign genes into host cells at random. The biological effects may therefore be related to the integration sites rather than the transgenes themselves, so it is hard to define gene function unambiguously. Targeted gene insertion by TALENs is a good HR-dependent approach to integrate genes of interest into genomes at given sites and avoid random insertions. Using this targeted gene insertion technology, researchers can select sites that are conducive to high levels of gene expression and integrate different genes at the same locus under the control of identical regulatory elements to detect subtle differences in gene function. Furthermore, it could be used to insert multiple genes at the same chromosomal locus to stack genes. This makes it easier to combine all of the genes of interest at the same locus and then transfer them into other germplasms by crossing to accelerate plant molecular breeding (Curtin et al., 2012; Tzfira et al., 2012). Targeted tagging is another example of targeted gene insertion. It specifically refers to inserting a tag or a reporter gene to label an endogenous gene. Zhang et al. (2013) used TALENs to generate an ALS-GFP fusion protein expressed in tobacco protoplasts.

Targeted gene replacement

Targeted gene replacement is normally obtained by an HR-dependent DNA repair pathway, in which TALENs and donor DNA templates containing sequences homologous to those flanking the break site are delivered to cells simultaneously. In this way DSBs induced by the TALENs can be immediately repaired using the donor DNA template and generate a specific change at a specific genomic location. Targeted gene replacements mediated by TALENs have been successfully used to modify predetermined sequences in yeast (Li et al., 2011), zebrafish (Bedell, 2012) and rice (Zhang et al., 2013).

Targeted chromosomal rearrangement

When two pairs of TALENs are delivered into cells at the same time, this will lead to more complex types of TGMs, including large deletions, inversions and translocations. Large deletions and inversions are usually generated by two pairs of TALENs targeted at two sites on the same chromosome; otherwise translocations are induced when two TALENs target different chromosomes. Because these modifications need two

DSBs to be introduced at the same time, relatively few chromosomal rearrangements have been obtained to date. Large deletions or inversions have been generated in livestock (Carlson et al., 2012), silkworm (Ma et al., 2012) and rice (Shan et al., 2013). The large chromosomal rearrangements obtained by simultaneous expression of two pairs of TALENs can be useful for a variety of purposes. For example, targeted deletions would make possible the selective removal of gene clusters and enables scientists to delete intergenic regions, introns, regulatory elements and noncoding RNAs.

TALEN-MEDIATED GENOME MODIFICATION IN PLANTS

Although TALEN technology benefits from the discovery of the DNA recognition code which TALEs have adopted to regulate effector gene expression in plants, the achievements of genome modification in plants are lagging behind those in other organisms such as zebrafish (Huang et al., 2011; Sander et al., 2011; Cade et al., 2012; Moore, 2012). Up to now, TALEN-mediated genome modification has been adopted in four plant species, *Arabidopsis* (Christian et al., 2010; Cermak et al., 2011; Li et al., 2012a), tobacco (Mahfouz et al., 2011; Li et al., 2012a; Zhang et al., 2013), rice (Li et al., 2012b; Shan et al., 2013) and *Brachypodium* (Shan et al., 2013).

Most of the resulting mutations reported in plants until now are NHEJ-dependent genome mutations. The initial research was carried out by Christian et al. (2010) in Voytas' laboratory, one year after the recognition code was deciphered. These workers assembled pairs of TALENs targeting the *Arabidopsis* gene *ADH1* and proved that they were active in a yeast assay (Christian et al., 2010). One year later Cermak et al. (2011) described Golden Gate Method and its reagents for efficiently assembling TALEN constructs with custom repeat arrays. The same *Arabidopsis* gene *ADH1* was successfully TALEN-targeted in *Arabidopsis* protoplasts using this method. In the same year, Zhu's group generated a *de novo* Hax3 Talebase hybrid nuclease (dHax3N) by fusing a modified dHax3 DBD sequence to a *Fok* I C terminal cleavage domain (Mahfouz et al., 2011). When transiently expressed in tobacco leaves, the modified Hax3N created DSBs in its artificial target sequence *in vivo*. Furthermore, Li et al. (2012a) assembled dTALEN heterodimers binding to the CDS of *AtPDS3* and demonstrated their cleavage activity *in vitro*.

The highpoint of the application of TALEN technology to crop improvement so far has been the production of disease-resistant rice by high-efficiency TALE-based gene editing (Li et al., 2012b). Bacterial blight is a devastating disease responsible for large losses of rice yields, and the main cause of the virulence was the TALE secreted by *Xanthomonas oryzae* pv. *oryzae* (Xoo), which transcriptionally activates specific rice disease-susceptibility (*S*) genes. Li et al. (2012b) used TALENs to edit a specific *S* gene, the sucrose-efflux transporter gene (*Os11N3*, also named *OsSWEET14*), by disrupting the effector-binding element (EBE) in its promoter region without changing its expression. TALENs targeted at an essential sequence covering the natural EBEs of *AvrXa7* and *PthXo3* were designed and transformed into rice cells. Several independent mutant lines with disrupted EBE sequences were obtained, and the progeny of some of them displayed resistance specific to *AvrXa7* and *PthXo3*, and morphologically normal phenotypes.

Targeted gene insertions and replacements have been accomplished by the Voytas laboratory using highly efficient TALE scaffolds optimized by employing tobacco protoplasts and TALENs targeting the acetolactate synthase (*ALS*) gene (Zhang et al., 2013). The high frequencies of targeted mutations (30%) and gene insertions (14%) hold promise of permitting genome modification by protoplast transformation without the need for selection or enrichment regimes. In addition, when TALENs were co-transformed with donor DNA carrying a 6-bp variant of *surA* or *surB*, targeted gene replacements (4%) in calli were achieved. The protoplast SSA (Single-strand Annealing) assay has been shown to provide a reliable means of assessing TALEN activity at endogenous chromosomal target sites. The Voytas's laboratory has provided plant biologists with an efficient plant genome engineering system using TALENs, including the Golden Gate assembly method (Cermak et al., 2011), optimal scaffolds of increased effectiveness (Zhang et al., 2013) and a nuclease activity validation method using protoplasts (Zhang et al., 2013). Their work thus offers hope of being able to perform plant genome engineering in general molecular biology labs.

Large scale highly efficient targeted gene modification by TALENs in rice and *Brachypodium* has been achieved in our laboratory (Shan et al., 2013). We obtained targeted gene knockouts of four rice genes and eight *Brachypodium* genes, with mutation rates reaching >30% as measured both by restriction enzyme digestion assays and sequencing. Our reagents and cloning processes are seamlessly integrated into a published Golden Gate TALEN engineering platform, which has been used by more the 800 laboratories worldwide (Pennisi, 2012). Instead of some laborious plant transformation procedure, a rapid, accurate and reliable protoplast transient assay, based on PCR/restriction enzyme screening, was used to quickly screen active nuclease activities independently at each endogenous target site in rice and *Brachypodium*. The active TALENs were subsequently transformed into embryonic cells of rice or *Brachypodium* using the *Agrobacterium* transformation method. We identified in total of 127 mutant sequences in both rice and *Brachypodium*, and each was characterized as a small deletion, insertion, or nucleotide substitution. Most mutations were small deletions ranging from 1 to 20 bp and all of the mutations occurred in the spacer region between the TALEN binding sites. These high frequencies allowed us to isolate large genomic deletions (e.g., 1.3 kb) by simultaneous expression of two pairs of TALENs. This was the first report that demonstrated the production of genomic deletions of large, predetermined endogenous DNA segments in a targeted fashion in plants. So far, nearly 70 TALEN pairs targeting 33 rice genes and 18 *Brachypodium* genes have been successfully constructed and validated in our laboratory (unpublished data). In addition, preliminary experiments show that TALENs can induce indel mutations that disrupt genes in *Arabidopsis*, petunia, cotton, maize and wheat (unpublished

data). It will not be surprising if, in the near future, TALEN technology is extended to potato, soybean, sorghum, oilseed rapes and other plants species or crops of commercial value.

CONCLUSIONS AND PERSPECTIVES

TALEN mediated targeted genome modification is rapidly becoming a powerful tools for genome engineering. In the last three years there has been an explosion in the number and diversity of applications of this technology (Joung and Sander, 2012; Marx, 2012; Mussolino and Cathomen, 2012; Pennisi, 2012). However TALEN-mediated TGM in plants is far behind that in other organisms. Several challenges remain to be addressed if these technologies are to be used routinely in plants. First, the efficiency of specific DNA targeting of TALENs has to be increased. Although truncated TALENs have been efficient in creating targeted gene knockouts in rice and *Brachypodium* (Shan et al., 2013; Zhang et al., 2013), the efficiencies of TALENs tested in our laboratory varied substantially. Some reached 100%, while about 50% of TALEN pairs had no detectable nuclease activity (unpublished data). Hence, more efficient TALENs that can be used in various plants are urgently needed. Many methods, including adopting scaffolds with modified amino acids, developing more efficient *Fok* I variants, selecting suitable linkers between TALE and *Fok* I, and optimizing the spacer length between the two monomers, hold great promise of increasing TALEN efficiency and should be tried out. Furthermore, a major problem preoccupying researchers is that we cannot predict the TALEN activity at specific sites. So another valuable contribution would be to develop some general rules for improving TALEN efficiency, and this might require collecting more information about the TALENs that have been tried out in various organisms.

Secondly, methods for TALEN delivery into cells need to be developed for different plants and genotypes. TALEN-mediated genome engineering in plants requires efficient genetic transformation. Since some plants, such as wheat, are described as recalcitrant to transformation, to realize TGMs in these plants, efficient genetic transformation protocols are needed.

Thirdly, our understanding of homologous recombination should be improved. Since DNA repair by HR is usually limited by a high rate of NHEJ, a thorough understanding of DNA repair mechanisms and the discovery of useful methods for enhancing HR are needed to achieve insertions, replacements, tagging and stacking, and particularly for introducing new traits into crop plants.

Finally, the application of the TALEN approach should be broadened to more plants, particularly to important crop species and cultivars. This depends not only on the development of TALENs but also on the adaptation and modification of existing tools and methods for TALEN validation and delivery in plants. And researchers should expand TGMs from TALENs to other TALE-based technologies, by combining TALEs with other functional domains such as repressors, activators, recombinases, nickases and methylases (Mahfouz and Li, 2011; Curtin et al., 2012; Joung and Sander, 2012). These will open up more possibilities for manipulating genomes at specific sites, and broaden our ability to regulate genes by different routes.

Although there are still problems limiting their application in plants, TALENs represent a breakthrough technology in genome engineering (2011; 2012; Baker, 2012; Joung and Sander, 2012). TALEN-induced genome modification in plants offers more possibilities of investigating sophisticated gene functions directly in planta. At the same time, it provides an efficient tool for genome engineering − for example, TALEN-mediated genome modification is much more rapid than traditional crop breeding. Furthermore, TALEN technology has the potential to allay concerns about the commercialization of genetically modified crops because foreign DNA is not involved and it gives rise to marker-free varieties as well as targeted mutations at clearly defined sites. Therefore we can anticipate that plant varieties generated by genome engineering will be regulated by governmental agencies in ways different from traditional transgenic plants (Kuzma and Kokotovich, 2011). Thus TALENs, act as customizable molecular DNA scissors, and open the way to the next generation of genome engineering in plants.

ACKNOWLEDGEMENTS

We thank Q. Shan, Z. Liang and K. Zhang in our laboratory for their helpful discussions and technical supports. This work was supported by the National Natural Science Foundation of China (Grant Nos. 201263, 383601 and 31200273).

REFERENCES

2011. Method of the year 2011. Nat. Methods 9, 1.
2012. The runners-up. Science 338, 1525−1532.
Baker, M., 2012. Gene-editing nucleases. Nat. Methods 9, 23−26.
Bedell, V.M., 2012. *In vivo* genome editing using a high-efficiency TALEN system. Nature. http://dx.doi.org/10.1038/nature11537.
Bibikova, M., Golic, M., Golic, K.G., Carroll, D., 2002. Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. Genetics 161, 1169−1175.
Boch, J., Bonas, U., 2010. *Xanthomonas* AvrBs3 family-type III effectors: discovery and function. Annu. Rev. Phytopathol. 48, 419−436.
Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., Lahaye, T., Nickstadt, A., Bonas, U., 2009. Breaking the code of DNA binding specificity of TAL-type III effectors. Science 326, 1509−1512.
Bogdanove, A.J., Schornack, S., Lahaye, T., 2010. TAL effectors: finding plant genes for disease and defense. Curr. Opin. Plant. Biol. 13, 394−401.
Bogdanove, A.J., Voytas, D.F., 2011. TAL effectors: customizable proteins for DNA targeting. Science 333, 1843−1846.
Briggs, A.W., Rios, X., Chari, R., Yang, L., Zhang, F., Mali, P., Church, G.M., 2012. Iterative capped assembly: rapid and scalable synthesis of repeat-module DNA such as TAL effectors from individual monomers. Nucleic Acids Res. 40, e117.
Cade, L., Reyon, D., Hwang, W.Y., Tsai, S.Q., Patel, S., Khayter, C., Joung, J.K., Sander, J.D., Peterson, R.T., Yeh, J.R., 2012. Highly efficient generation of heritable zebrafish gene mutations using homo- and heterodimeric TALENs. Nucleic Acids Res. 40, 8001−8010.
Capecchi, M.R., 2005. Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. Nat. Rev. Genet. 6, 507−512.
Carlson, D.F., Tan, W., Lillico, S.G., Stverakova, D., Proudfoot, C., Christian, M., Voytas, D.F., Long, C.R., Whitelaw, C.B., Fahrenkrug, S.C.,

2012. Efficient TALEN-mediated gene knockout in livestock. Proc. Natl. Acad. Sci. USA 109, 17382−17387.

Carroll, D., 2011. Genome engineering with zinc-finger nucleases. Genetics 188, 773−782.

Cermak, T., Doyle, E.L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J.A., Somia, N.V., Bogdanove, A.J., Voytas, D.F., 2011. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res. 39, e82.

Christian, M., Cermak, T., Doyle, E.L., Schmidt, C., Zhang, F., Hummel, A., Bogdanove, A.J., Voytas, D.F., 2010. Targeting DNA double-strand breaks with TAL effector nucleases. Genetics 186, 757−761.

Curtin, S.J., Voytas, D.F., Stupar, R.M., 2012. Genome engineering of crops with designer nucleases. Plant Genome J. 5, 42−50.

Curtin, S.J., Zhang, F., Sander, J.D., Haun, W.J., Starker, C., Baltes, N.J., Reyon, D., Dahlborg, E.J., Goodwin, M.J., Coffman, A.P., Dobbs, D., Joung, J.K., Voytas, D.F., Stupar, R.M., 2011. Targeted mutagenesis of duplicated genes in soybean with zinc-finger nucleases. Plant Physiol. 156, 466−473.

Dahlem, T.J., Hoshijima, K., Jurynec, M.J., Gunther, D., Starker, C.G., Locke, A.S., Weis, A.M., Voytas, D.F., Grunwald, D.J., 2012. Simple methods for generating and detecting locus-specific mutations induced with TALENs in the zebrafish genome. PLoS Genet. 8, e1002861.

de Pater, S., Neuteboom, L.W., Pinas, J.E., Hooykaas, P.J., van der Zaal, B.J., 2009. ZFN-induced mutagenesis and gene-targeting in *Arabidopsis* through *Agrobacterium*-mediated floral dip transformation. Plant Biotechnol. J. 7, 821−835.

DeFrancesco, L., 2011. Move over ZFNs. Nat. Biotechnol. 29, 681−684.

Deng, D., Yan, C., Pan, X., Mahfouz, M., Wang, J., Zhu, J.K., Shi, Y., Yan, N., 2012a. Structural basis for sequence-specific recognition of DNA by TAL effectors. Science 335, 720−723.

Deng, D., Yin, P., Yan, C., Pan, X., Gong, X., Qi, S., Xie, T., Mahfouz, M., Zhu, J.K., Yan, N., Shi, Y., 2012b. Recognition of methylated DNA by TAL effectors. Cell Res. 22, 1502−1504.

Doyle, E.L., Booher, N.J., Standage, D.S., Voytas, D.F., Brendel, V.P., Vandyk, J.K., Bogdanove, A.J., 2012. TAL Effector-Nucleotide Targeter (TALE-NT) 2.0: tools for TAL effector design and target prediction. Nucleic Acids Res. 40, W117−W122.

Halfter, U., Morris, P.C., Willmitzer, L., 1992. Gene targeting in *Arabidopsis thaliana*. Mol. Gen. Genet. 231, 186−193.

Hockemeyer, D., Wang, H., Kiani, S., Lai, C.S., Gao, Q., Cassady, J.P., Cost, G.J., Zhang, L., Santiago, Y., Miller, J.C., Zeitler, B., Cherone, J.M., Meng, X., Hinkley, S.J., Rebar, E.J., Gregory, P.D., Urnov, F.D., Jaenisch, R., 2011. Genetic engineering of human pluripotent cells using TALE nucleases. Nat. Biotechnol. 29, 731−734.

Huang, P., Xiao, A., Zhou, M., Zhu, Z., Lin, S., Zhang, B., 2011. Heritable gene targeting in zebrafish using customized TALENs. Nat. Biotechnol. 29, 699−700.

Joung, J.K., Sander, J.D., 2012. TALENs: a widely applicable technology for targeted genome editing. Nat. Rev. Mol. Cell Biol. 14, 49−55.

Kuzma, J., Kokotovich, A., 2011. Renegotiating GM crop regulation. EMBO Rep. 12, 883−888.

Lei, Y., 2012. Efficient targeted gene disruption in *Xenopus embryos* using engineered transcription activator-like effector nucleases (TALENs). Proc. Natl. Acad. Sci. USA 109, 17484−17489.

Li, L., Piatek, M.J., Atef, A., Piatek, A., Wibowo, A., Fang, X., Sabir, J.S., Zhu, J.K., Mahfouz, M.M., 2012a. Rapid and highly efficient construction of TALE-based transcriptional regulators and nucleases for genome modification. Plant Mol. Biol. 78, 407−416.

Li, T., Huang, S., Zhao, X., Wright, D.A., Carpenter, S., Spalding, M.H., Weeks, D.P., Yang, B., 2011. Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes. Nucleic Acids Res. 39, 6315−6325.

Li, T., Liu, B., Spalding, M.H., Weeks, D.P., Yang, B., 2012b. High-efficiency TALEN-based gene editing produces disease-resistant rice. Nat. Biotechnol. 30, 390−392.

Liu, J., Li, C., Yu, Z., Huang, P., Wu, H., Wei, C., Zhu, N., Shen, Y., Chen, Y., Zhang, B., Deng, W.M., Jiao, R., 2012. Efficient and specific modifications of the *Drosophila* genome by means of an easy TALEN strategy. J. Genet. Genomics 39, 209−215.

Ma, S., Zhang, S., Wang, F., Liu, Y., Liu, Y., Xu, H., Liu, C., Lin, Y., Zhao, P., Xia, Q., 2012. Highly efficient and specific genome editing in silkworm using custom TALENs. PLoS ONE 7, e45035.

Mahfouz, M.M., Li, L., 2011. TALE nucleases and next generation GM crops. GM Crops 2, 99−103.

Mahfouz, M.M., Li, L., Shamimuzzaman, M., Wibowo, A., Fang, X., Zhu, J.K., 2011. *De novo*-engineered transcription activator-like effector (TALE) hybrid nuclease with novel DNA binding specificity creates double-strand breaks. Proc. Natl. Acad. Sci. USA 108, 2623−2628.

Mak, A.N., Bradley, P., Cernadas, R.A., Bogdanove, A.J., Stoddard, B.L., 2012. The crystal structure of TAL effector PthXo1 bound to its DNA target. Science 335, 716−719.

Marx, V., 2012. Genome-editing tools storm ahead. Nat. Methods 9, 1055−1059.

Miller, J.C., Tan, S., Qiao, G., Barlow, K.A., Wang, J., Xia, D.F., Meng, X., Paschon, D.E., Leung, E., Hinkley, S.J., Dulay, G.P., Hua, K.L., Ankoudinova, I., Cost, G.J., Urnov, F.D., Zhang, H.S., Holmes, M.C., Zhang, L., Gregory, P.D., Rebar, E.J., 2011. A TALE nuclease architecture for efficient genome editing. Nat. Biotechnol. 29, 143−148.

Moore, F.E., 2012. Improved somatic mutagenesis in zebrafish using transcription activator-like effector nucleases (TALENs). PLoS ONE 7, e37877.

Moscou, M.J., Bogdanove, A.J., 2009. A simple cipher governs DNA recognition by TAL effectors. Science 326, 1501.

Mussolino, C., Cathomen, T., 2012. TALE nucleases: tailored genome engineering made easy. Curr. Opin. Biotechnol. 23, 644−650.

Osakabe, K., Osakabe, Y., Toki, S., 2010. Site-directed mutagenesis in *Arabidopsis* using custom-designed zinc finger nucleases. Proc. Natl. Acad. Sci. USA 107, 12034−12039.

Paszkowski, J., Baur, M., Bogucki, A., Potrykus, I., 1988. Gene targeting in plants. EMBO J. 7, 4021−4026.

Pennisi, E., 2010. Sowing the seeds for the ideal crop. Science 327, 802−803.

Pennisi, E., 2012. The tale of the TALEs. Science 338, 1408−1411.

Remy, S., Tesson, L., Menoret, S., Usal, C., Scharenberg, A.M., Anegon, I., 2010. Zinc-finger nucleases: a powerful tool for genetic engineering of animals. Transgenic Res. 19, 363−371.

Reyon, D., Khayter, C., Regan, M.R., Joung, J.K., Sander, J.D., 2012a. Engineering designer transcription activator-like effector nucleases (TALENs) by REAL or REAL-Fast assembly. Curr. Protoc. Mol. Biol. Chapter 12, Unit 12 15. http://dx.doi.org/10.1002/0471142727.mb1215s100.

Reyon, D., Tsai, S.Q., Khayter, C., Foden, J.A., Sander, J.D., Joung, J.K., 2012b. FLASH assembly of TALENs for high-throughput genome editing. Nat. Biotechnol. 30, 460−465.

Rouet, P., Smih, F., Jasin, M., 1994. Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. Proc. Natl. Acad. Sci. USA 91, 6064−6068.

Sander, J.D., Cade, L., Khayter, C., Reyon, D., Peterson, R.T., Joung, J.K., Yeh, J.R., 2011. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. Nat. Biotechnol. 29, 697−698.

Scherer, S., Davis, R.W., 1979. Replacement of chromosome segments with altered DNA sequences constructed *in vitro*. Proc. Natl. Acad. Sci. USA 76, 4951−4955.

Schmid-Burgk, J.L., Schmidt, T., Kaiser, V., Honing, K., Hornung, V., 2012. A ligation-independent cloning technique for high-throughput assembly of transcription activator-like effector genes. Nat. Biotechnol. 31, 76−81.

Shan, Q., Wang, Y., Chen, K., Liang, Z., Li, J., Zhang, Y., Zhang, K., Liu, J., Voytas, D.F., Zheng, X., Zhang, Y., Gao, C., 2013. Rapid and efficient gene modification in rice and *Brachypodium* using TALENs. Mol. Plant. http://dx.doi.org/10.1093/mp/sss162.

Shukla, V.K., Doyon, Y., Miller, J.C., deKelver, R.C., Moehle, E.A., Worden, S.E., Mitchell, J.C., Arnold, N.L., Gopalan, S., Meng, X., Choi, V.M., Rock, J.M., Wu, Y.Y., Katibah, G.E., Zhifang, G., McCaskill, D., Simpson, M.A., Blakeslee, B., Greenwalt, S.A., Butler, H.J., Hinkley, S.J., Zhang, L., Rebar, E.J., Gregory, P.D., Urnov, F.D., 2009. Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. Nature 459, 437−441.

Streubel, J., Blucher, C., Landgraf, A., Boch, J., 2012. TAL effector RVD specificities and efficiencies. Nat. Biotechnol. 30, 593−595.

Tesson, L., 2011. Knockout rats generated by embryo microinjection of TALENs. Nat. Biotechnol. 29, 695−696.

Tong, C., Huang, G., Ashton, C., Wu, H., Yan, H., Ying, Q.L., 2012. Rapid and cost-effective gene targeting in rat embryonic stem cells by TALENs. J. Genet. Genomics 39, 275−280.

Townsend, J.A., Wright, D.A., Winfrey, R.J., Fu, F., Maeder, M.L., Joung, J.K., Voytas, D.F., 2009. High-frequency modification of plant genes using engineered zinc-finger nucleases. Nature 459, 442−445.

Tzfira, T., Weinthal, D., Marton, I., Zeevi, V., Zuker, A., Vainstein, A., 2012. Genome modifications in plant cells by custom-made restriction enzymes. Plant Biotechnol. J. 10, 373−389.

Urnov, F.D., Miller, J.C., Lee, Y.L., Beausejour, C.M., Rock, J.M., Augustus, S., Jamieson, A.C., Porteus, M.H., Gregory, P.D., Holmes, M.C., 2005. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature 435, 646−651.

Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S., Gregory, P.D., 2010. Genome editing with engineered zinc finger nucleases. Nat. Rev. Genet. 11, 636−646.

Valton, J., Dupuy, A., Daboussi, F., Thomas, S., Marechal, A., Macmaster, R., Melliand, K., Juillerat, A., Duchateau, P., 2012. Overcoming transcription activator-like effector (TALE) DNA binding domain sensitivity to cytosine methylation. J. Biol. Chem. 287, 38427−38432.

Wang, Z., 2012. An integrated Chip for the high-throughput synthesis of transcription activator-like effectors. Angew. Chem. Int. Ed. Engl. 51, 8505−8508.

Weber, E., Gruetzner, R., Werner, S., Engler, C., Marillonnet, S., 2011. Assembly of designer TAL effectors by Golden Gate cloning. PLoS ONE 6, e19722.

Wood, A.J., 2011. Targeted genome editing across species using ZFNs and TALENs. Science 333, 307.

Wright, D.A., Townsend, J.A., Winfrey Jr., R.J., Irwin, P.A., Rajagopal, J., Lonosky, P.M., Hall, B.D., Jondle, M.D., Voytas, D.F., 2005. High-frequency homologous recombination in plants mediated by zinc-finger nucleases. Plant J. 44, 693−705.

Xiao, A., Wu, Y., Yang, Z., Hu, Y., Wang, W., Zhang, Y., Kong, L., Gao, G., Zhu, Z., Lin, S., Zhang, B., 2013. EENdb: a database and knowledge base of ZFNs and TALENs for endonuclease engineering. Nucleic Acids Res. 41, D415−D422.

Zhang, F., Cong, L., Lodato, S., Kosuri, S., Church, G.M., Arlotta, P., 2011. Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. Nat. Biotechnol. 29, 149−153.

Zhang, F., Maeder, M.L., Unger-Wallace, E., Hoshaw, J.P., Reyon, D., Christian, M., Li, X., Pierick, C.J., Dobbs, D., Peterson, T., 2010. High frequency targeted mutagenesis in *Arabidopsis thaliana* using zinc finger nucleases. Proc. Natl. Acad. Sci. USA 107, 12028−12033.

Zhang, Y., Zhang, F., Li, X., Baller, J.A., Qi, Y., Starker, C.G., Bogdanove, A.J., Voytas, D.F., 2013. Transcription activator-like effector nucleases enable efficient plant genome engineering. Plant Physiol. 161, 20−27.