Chapter 12

Genetic Engineering of Plants Using Zn Fingers, TALENs, and CRISPRs

A. Bilichak, F. Eudes

Agriculture and Agri-Food Canada, Lethbridge, AB, Canada

Chapter Outline

1. INTRODUCTION

The improvement of crop traits has been an ongoing practice since plant domestication. A conventional breeding technique utilizes an available pool of natural genetic variation combined with extensive backcrossing to introduce traits into an elite background. The existence of valuable alleles in nature and the inability to introduce traits selectively limit the outcome of this approach.

The discovery of X-ray mutagenesis in the 1920s [1,2] has initiated the development of mutation breeding for the artificial introduction of new traits in crops of interest. In the process, the mutagenized population of plants was screened using forward genetic approaches for the direct identification of specific phenotypes. This method has a significant drawback because most of the random mutations are recessive, and the polyploidy of crop species can mask any phenotypic effects resulted from a given sequence mutation [3]. The lack of technology to target mutations to predefined positions in the genome also impeded the utilization of reverse genetic screening for a fast and efficient linkage between a gene and a phenotype. Hence, further progress of plant biotechnology and breeding necessitates the discovery of new tools for targeted genetic engineering.

Genetic engineering is an umbrella term that covers a precise modification of the genome by means of the targeted insertion, replacement, or editing of the selected locus/loci (Fig. 12.1). Historically, homologous recombination (HR) was the method of choice to achieve gene targeting (GT) in model organisms [4]. Efficient HR in eukaryotes has been overall limited to yeast, chicken DT40 cells, mouse embryonic stem (ES) cells, and moss *Physcomitrella patens* (reviewed in Refs. [5,6]). At the dawn of plant biotechnology, a major impediment to genetic engineering in vascular plants was the limited frequencies of HR ranging from 10^{-4} to 10^{-6} (reviewed in Ref. [7]). The implementation of positive/negative selection markers [8,9] and the labor-intensive screening of the generated transgenic plants had to be performed to identify putative GT events in the plant population [7]. The pioneer studies on the utilization of a rare cutting yeast enzyme I-*Sce*-I in plants and animals have revealed that the cleavage of DNA at the artificially created endogenous position increases the rate of HR and GT events by 1000 folds or more at this locus [10–13]. Most of the DNA double-strand breaks (DSBs) in plants are repaired through the error-prone nonhomologous end joining (NHEJ) pathway that results in the introduction of insertion/deletions at the cut site [14]. This natural effect is utilized to produce lines with the desired gene knockouts in a time- and cost-efficient manner.

Genome Stability. http://dx.doi.org/10.1016/B978-0-12-803309-8.00012-4

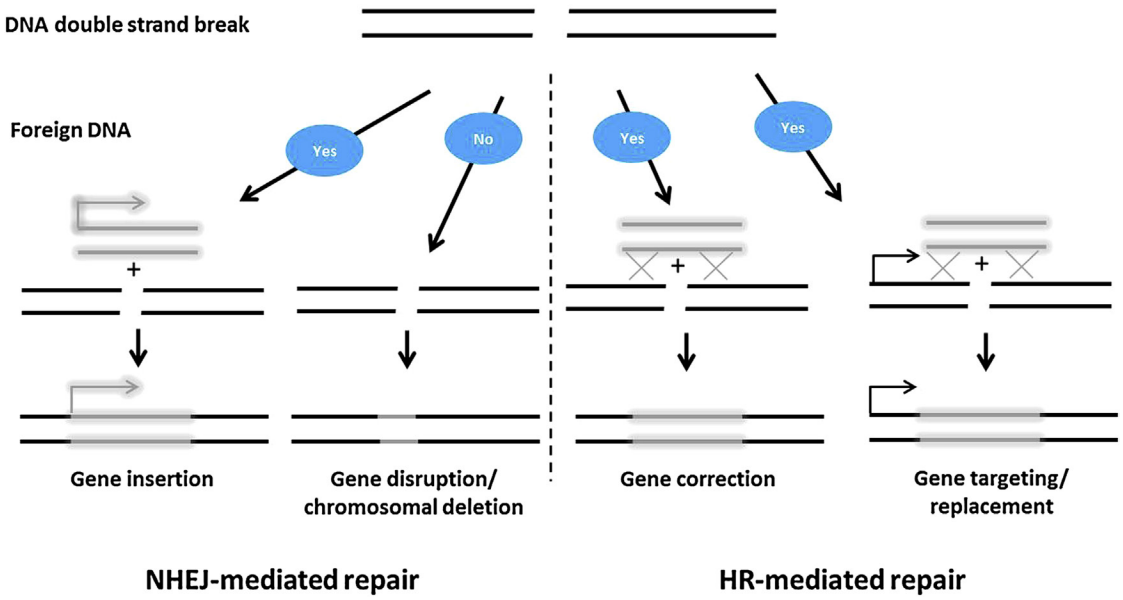

FIGURE 12.1 **Possible strategies for genome editing in plants using designed endonucleases.** The induction of double-strand breaks by endonucleases typically increases the frequency of genome editing by hundred times as compared to those resulted from spontaneous homologous recombination. Supplementing the donor DNA (shown in gray) either with or without homology to the endogenous region can lead to different outcomes depending on the DNA repair pathway involved.

The generation of targeted DSBs requires a protein or nucleoprotein complex that can be designed to bind to any sequence of interest [15]. A fusion of a programmable DNA-binding motif to the nonspecific endonuclease domain allows for a precise introduction of DSBs at the preselected positions [16]. In the late 1990s, the first artificial endonucleases appeared on the horizon that set a stage for the rapid development of novel enzymes with a specific cleavage activity called the designed or engineered endonucleases. Currently, four types of engineered nucleases are used for genome editing: engineered homing endonucleases/meganucleases (EMNs), zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9). All of them have been successfully used in plants to introduce modifications at the predefined positions in the genome. Nevertheless, challenges with the design, verification, and prohibitive licensing fees associated with some of the engineered endonucleases made their utilization less frequent as compared to other endonucleases. In particular, as of 2016, TALENs and CRISPR/Cas9 are the most widely used technologies in plants [17]. In this chapter, we provide a brief overview of the current status of the genome-editing technology in plants using the designed endonucleases and the future perspective of the possible technology application in plant genetic engineering.

2. ZINC FINGER NUCLEASES FOR GENOME ENGINEERING OF PLANTS

The Cys2–His2 zinc-finger motif is one of the most common types of DNA-binding domains present in eukaryotes. In addition, it is observed in almost half of transcription factors in humans [18–20]. The invention of ZFNs was a gradual process that included deciphering the interaction of zinc-finger motifs with DNA and the examination of the most efficient strategy of fusion of a DNA-binding domain to the nonspecific endonuclease *Fok*I (Fig. 12.2). The *Fok*I protein is a type II restriction enzyme produced by *Flavobacterium okeanokoites* [21]. The N-terminal end of the protein is a DNA-binding motif, and the C-terminal end acts as a nonspecific cleavage domain. Upon binding to its target sequence and in the presence of divalent metal ions, the *Fok*I enzyme dimerizes [22]. The *Fok*I nonspecific cleavage domain is also used in chimeric endonucleases, such as ZFNs, TALENs, and in the specialized CRISPR/dCas9 enzymes.

A single zinc-finger unit consists of three or four binding modules, and each module recognizes a nucleotide (nt) triplet. Two ZFN monomers can bind to the unique 18–24 bp-long sequences spaced by a 5–6 bp gap between them. Upon *Fok*I dimerization, DSB is created with 4–5 bp 5′-overhangs [23]. Since the first demonstration of the yellow gene disruption in a fruit fly in 2002 [24], various ZFNs have been applied for genome editing in a number of plants, including *Arabidopsis*, tobacco, maize, and soybean [25–29].

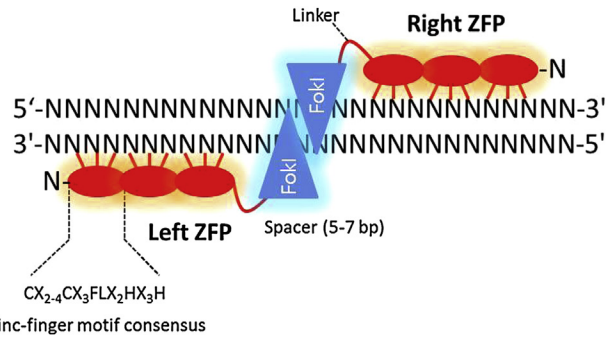

FIGURE 12.2 **Schematic representation of a zinc-finger nuclease (ZFN) protein.** Each ZFN consists of a zinc-finger protein (ZFP) at the N-terminus and a *Fok*I nuclease domain at the C-terminus. ZFN typically can target 18–36 bp long sequences. In the zinc-finger-motif consensus, X represents any amino acid.

2.1 Zinc Finger Nucleases Application in Model Plant Species

The first pioneering studies on ZFN-induced targeted mutagenesis in plants involved the modification of previously integrated transgenes carrying the ZFN cleavage sites in *Arabidopsis* [30–32]. Mutation frequencies ranging from 2% [31] to as high as 19.6% were observed in *Arabidopsis* depending on the ZFN-induction system used (constitutive versus inducible expression). A nontransgenic virus-based ZFN delivery in tobacco and petunia plants resulted in a targeted modification of reporter construct in somatic cells that was stably inherited in the following generation [33]. In most of the cases, the authors observed simple deletions at the target sites of 1–80 bp, thus reinforcing the hypothesis that imprecise NHEJ repair prevails in higher plants [30].

Some examples of endogenous genomic loci mutations mediated by ZFNs in *Arabidopsis* include the disruption of *ABAINSENSITIVE-4* (*ABI4*) [27], *ALCOHOL DEHYDROGENASE-1* (*ADH1*), and *TRANSPARENT TESTA-4* (*TT4*) genes [28]. In both of the studies, the inducible promoters were used for the activation of the previously integrated ZFN constructs. Upon induction, the observed mutation frequencies in somatic cells were 3%, 7%, and 16% for *ABI4*, *ADH1*, and *TT4*, respectively. Mutations were stably transmitted to the progeny, and the associated phenotype was observed for all genes. Curiously, for both of the genes, *ADH1* and *TT4*, homozygous mutants in the T1 generation were recovered, suggesting simultaneous biallelic mutations. At the same time, no potential off-target effects were observed in the edited plants [28].

A broader application of NHEJ-mediated gene disruption includes the replacement of an endogenous locus preceded by its cleavage at the 5′- and 3′-termini using ZFNs. Successful deletions of 2.8 and 4.3 kb fragments at the transgene loci were reported in tobacco plants [34,35]. The expression of ZFN in transgenic tobacco plants containing a GREEN FLUORESCENT PROTEIN (GFP) recombination construct with a 2.8 kb interrupting DNA sequence and a ZFN cleavage site resulted in the targeted DSB formation, recombination between GFP gene fragments, and deletion of the intervening 2.8 kb sequence. Moreover, the successful targeted deletions, inversions, and duplications of multiple gene clusters mediated by ZFNs have also been reported in *Arabidopsis* [36]. The simultaneous deletion of eight resistance (*R*) genes that compose a *RPP4* gene cluster was achieved by the inducible expression of ZFNs that targeted the regions of 55 kb apart [36]. The frequency of deletions in somatic cells was about 1%. Furthermore, the authors have even achieved deletions larger than 9 Mb on the chromosome 1 with the frequency of less than 1%. The feasibility of targeted deletions of large chromosomal regions offers an opportunity of a precise removal of a particular trait when it is regulated by a few clustered genes in crops. In addition, a gene cluster can be replaced with the genes of interest when the HR-mediated integration is engaged.

An additional precision of genome engineering can be achieved when ZFN cassettes are co-delivered into plant cells with the donor DNA that has homology to the endogenous sequence flanking the ZFN cut site. In this case, the HR pathway can be involved in DSB repair, and the donor DNA can be used as a template in the process of synthesis-dependent strand annealing [37]. Sequence modifications from the donor DNA can be copied into the targeted cut site with modifications that can vary from single- to few base-pair modifications (ie, gene editing) to the integration of complete transgene expression cassettes (ie, site-specific integration) [3]. Since the frequency of HR repair in somatic plant cells appears to be extremely low, the identification and isolation of such modifications is usually achieved by applying a selection pressure [3]. In one of the examples, specific mutations of the *SULFONYLUREA RECEPTOR* genes *SuRA* and *SuRB* in tobacco render cells insensitive to imidazolinone and sulfonylurea herbicides [26]. The co-delivery of ZFNs and the donor DNA template for the correction of *SuRA* and *SuRB* genes into tobacco protoplasts resulted in the recovery of herbicide-resistant calli at the frequency of 2%. Moreover, mutations as far as 1.3 kb from the ZFN cleavage site were obtained, suggesting that plant

genes can be edited even when the DNA sequence excludes the ZFN recognition sites near the desired locus of modifications [26]. Similarly, two specific point mutations in a *PROTOPORPHYRINOGEN OXIDASE* (*PPOX*) gene of *Arabidopsis* confer the plant's resistance to the herbicide butafenacil [38]. Floral dip transformation of wild-type plants and plants that constitutively express ZFN was performed using *Agrobacterium* carrying a binary vector. The plasmid contained a donor template with the *PPO* gene missing the 5′ coding region but having two necessary mutations to confer resistance to the herbicide butafenacil in the edited plants. Selection of T1 plants on butafenacil yielded GT frequencies of 0.8×10^{-3} and 3.1×10^{-3} per transformation event, in wild-type and ZFN lines, respectively.

The future advancement of gene-editing technology through HR will require the development of tools for high-throughput screening of the generated plant populations. This will allow a selection-free elimination of wild-type plants in order to find the desired modification [3].

2.2 Zinc Finger Nucleases Application in Crops

Genome editing in crops presents a particular challenge because it relies on the availability of a highly efficient transformation method and the ability to design a unique engineered endonuclease for targeting a distinct locus, or loci, in the complex polyploid genome. The first successful report of genome editing in crops has been published in 2009. It involved the ZFN-mediated disruption of an *IPK1* gene that encodes an inositol-1,3,4,5,6-penta-kisphosphate 2-kinase, an enzyme that catalyzes the final step in phytate biosynthesis in maize seeds [25]. Phytate accounts for 75% of the total seed phosphorus and is an antinutritional component of feed grains that contributes to the environmental pollution through the waste stream. Reducing the level of phytate is agriculturally important for both increasing the bioavailability of phosphorus in corn grains and decreasing the negative environmental impact. Four ZFN pairs designed to cut *IPK1* at two positions in exon 2 were transformed into embryogenic callus of maize using a whisker-mediated DNA delivery [39]. Along with the ZFN cassettes, two HR repair templates were transformed that contained short homology arms to the *IPK1* gene and either an autonomous herbicide-tolerance gene expression sequence (*PAT*) or a nonautonomous donor that relied on a precise integration under the endogenous *IPK1* promoter for the expression of the marker gene. The frequencies of successful GT events ranged from 3.4% to 100%, depending on the ZFN pair used and the donor template. No off-target mutations were observed at the noncognate homologous sites in T0 plants carrying GT events at the *IPK1* gene. The effect of gene disruption on *IPK1* expression was transmitted through two rounds of meiosis, and the edited plants had a significant number of seeds with reduced phytate levels and a concomitant increase in inorganic phosphate as compared to plants with random integration of the donor template [25].

In another example, a targeted mutagenesis of a transgene and nine endogenous soybean (*Glycine max*) genes was performed using ZFNs [29]. Soybean has a highly duplicated paleopolyploid genome that jeopardized the development of ZFNs which recognize distinctive sequences in the genome. A number of ZFN constructs were constructed to target either unique or duplicated paralogs of epigenetic-related genes. Following *Agrobacterium rhizogenes*-mediated hairy-root transformation, somatic mutations were detected for the following genes: *DICER-LIKE1a* (*DCL1a*), *DCL1b*, *DCL4a*, *DCL4b*, *RNA-DEPENDENT RNA POLYMERASE 6a* (*RDR6a*), *RDR6b*, and *HUA ENHANCER 1a* (*HEN1a*). The whole-plant transformation of soybean using a cassette under the control of an estrogen-inducible promoter and encoding ZFNs targeting two paralogous genes, *DCL4a* and *DCL4b*, resulted in the recovery of three T0 plants from the hormone-treated explants. Sequence analysis of PCR-amplified products revealed that one of the plants had an adenine base insertion at the *DCL4a* locus, and another one had a two-base thymine and adenine insertion at the *DCL4b* locus. Both plants appeared to be heterozygous for the mutation. The plant with the *dcl4a* mutation exhibited phenotypic abnormalities, including aborted seed development. The *dcl4b* plant appeared to be normal and produced T1 progeny in which the *dcl4b* mutation segregated in a Mendelian fashion as1:2:1 [29]. These results provide the clear evidence that the designed endonucleases can be successfully implemented in the paleopolyploid crop species for a precise genome editing.

The development of a single crop variety with disease resistance, abiotic stress tolerance, yield enhancement, and quality traits requires the involvement of a labor- and resource-intensive introgression via conventional breeding. The appearance of tools for targeting DNA sequence to a selected locus may apparently eliminate problems of unpredicted cassette expression due to chromatin composition and segregation problems following meiosis. Combining two or more traits in one variety can now be achieved by molecular trait stacking in a single transgene locus. This can be done by using a transformation vector carrying the trait genes with homology sequences to the target region and a ZFN expression cassette designed to target the desired integration locus upon expression of an active protein. The proof-of-concept study has provided an example of successful on-demand transgene integration and trait stacking in the maize genome [40]. The authors used modular "trait landing pads" (TLPs) that flanked the herbicide-resistance gene, *pat*, and had ZFN target sites with sequences homologous to an incoming DNA. Separate cotransformation of transgenic plants with a donor DNA containing a second

herbicide-resistance gene, *aad1*, flanked by sequences homologous to the integrated TLP along with the corresponding ZFN expression construct allowed for the *aad1* transgene to be precisely integrated at TLP, directly adjacent to the *pat* transgene. The frequency of up to 5% in the embryo-derived transgenic events was achieved, and both herbicide-resistance genes co-segregated in the subsequent generations.

2.3 Potential Limitations of the Zinc Finger Nucleases Technology

Although the ZFN technology has proven itself as an efficient tool for the genome editing in a number of model and crop species, the design of multi–zinc finger modules is challenging due to complex interactions between amino acid residues and base pairs of the target sequence [41]. In addition, the assembly and testing of ZFNs is usually expensive. Moreover, the availability of endogenous targets is restricted due to a limited number of modules for the context-dependent assembly platform [42].

A major drawback of the broad usage of ZFNs in plants is a prohibitive licensing fee that restricts an access to the required design tools developed by the company Sangamo Bioscience [43].

3. TALENs FOR THE GENOME ENGINEERING OF PLANTS

Following pioneering studies on ZFNs, the genome engineering using TALENs in plants has progressed rapidly [44]. The transcription activator-like effector (TALE) DNA-binding domains were "borrowed" from plant pathogens in the genus *Xanthomonas* which deliver the proteins to plant cells during infection through the type III secretion pathway [45,46]. The TALE proteins can bind to the effector-specific DNA sequences and transcriptionally activate gene expression of host genes. This makes plants more susceptible to the pathogen attack in most of the cases. Binding of the TALE protein to DNA sequence is mediated by a middle region that contains 30 tandem repeats of a 33–35 amino acid–sequence motif. Each repeat has a mostly consistent amino acid sequence, with the exception of two adjacent amino acids (the repeat variable diresidue or RVD) at positions 12 and 13. Distinct RVDs within the repeats dictate the specificity of the repeat to recognize nts in the target sequence. In 2009, the cipher was decoded by two research groups who showed a clear relation between RVDs in the repeat domain and the nts in the target DNA sequence [45,47,48]. Using the current ZFN architecture, TALEs were fused to the catalytic domain of the *Fok*I restriction enzyme, and the resulting chimeric endonucleases also demonstrated a specific cleavage activity in the yeast LacZ assay [45] (Fig. 12.3). The off-target effects of TALENs seem to be fewer than those of ZFNs due to the longer target recognition site [7]. The assembly of TALENs has been simplified by the Golden Gate-based cloning method that allows directional and seamless assembly of multiple DNA fragments [49]. The availability of the tool kit along with the freely distributed module assembly plasmids allowed a number of groups to design and construct TALENs for the specific genome-editing objectives.

3.1 The Application of TALENs in the Model Plant Species

The in planta testing of designed TALENs was first performed in tobacco by transient cotransformation of a *uidA* reporter construct carrying the recognition sequence and the corresponding TALEN using *Agrobacterium* [50]. The cleavage at the

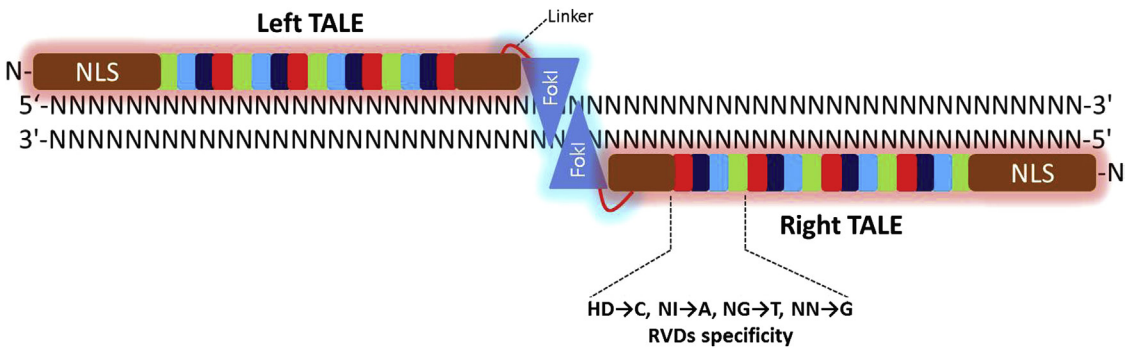

FIGURE 12.3 Schematic representation of a transcription activator-like effector nuclease (TALEN). Each monomer contains a DNA-binding domain at the amino terminus and a *Fok*I nuclease domain at the carboxyl terminus. Each TALE module (shown as small colored boxes) can recognize only one nucleotide through its 13th amino acid. Each TALE module typically contains 34 amino acids with the 12th and 13th residues being responsible for the specificity (repeated variable diresidues). A recognition pattern of modules is shown in the figure. *NLS*, a nuclear localization signal.

recognition sequence followed by a subsequent repair mediated by the cellular repair machinery would remove the stop codon in the reporter coding sequence and allowed for the expression of the *uidA* reporter. Following the co-delivery of both constructs into tobacco leaves, the authors observed blue sectors in the infiltration regions, and the resulting products of DSB repair were confirmed by sequencing [50]. Similarly, a transient expression of custom-designed TALEN targeting an *ALCOHOL DEHYDROGENASE1* (*ADH1*) gene in *Arabidopsis* resulted in the recovery of six independent mutations consisting of deletions ranging from 4 to 15 bp [51]. To assess the TALEN activityin planta, a yellow fluorescent protein (YFP)-based single-strand annealing (SSA) reporter construct has been developed [7]. The reporter has a TALEN recognition sequence flanked by a 255 bp direct repeat of the YFP-coding sequence. A successful cleavage of the construct by TALEN results in the recombination of homologous sequences and the reconstitution of a functional YFP gene. The co-delivery of both TALEN and the reporter construct into tobacco protoplasts allows for a fast screening of the TALEN activity using flow cytometry. The TALEN activities observed in the protoplast SSA assay demonstrated a high correlation with mutagenesis frequencies detected at the endogenous loci for the same TALENs. The mutagenesis efficiencies after TALEN delivery were in the range of 30% for an *ACETOLACTATE SYNTHASE* (*ALS*) gene that allowed the recovery of calli with targeted mutations without applying a selection pressure. In addition, 4% of calli showed an evidence of targeted gene replacement when a 322 bp donor molecule with 6 bp difference from the *ALS*-coding sequence was co-delivered with TALEN.

A stable integration of TALEN constructs designed to target separately five different genes in the *Arabidopsis* genome resulted in somatic mutagenesis frequencies ranging from 2% to 15% at the selected loci for all tested TALENs [52]. Furthermore, mutations were transmitted to the next generation at the rate of 1.5–12%. A stable germline transmission of somatic mutations in *Arabidopsis* caused by TALEN activity was also confirmed in a separate study [53]. The expression of TALENs under control of a shoot apical meristem–specific promoter resulted in targeting a *CLAVATA3* (*CLV3*) gene at the rate that allowed a recovery of biallelic mutants already in the T1 generation.

The successful application of TALENs in two monocot model species, rice and *Brachypodium*, demonstrated the utility of the tool for gene disruption in cereal crops [54]. When a stable *Agrobacterium*-mediated transformation of embryonic cells was performed with TALENs, the mutation frequencies of resistant calli were recovered at the rate from 3.8% to 100%, depending on TALEN and the species. Most of the mutations were small deletions ranging from 1 to 20 bp, and biallelic modifications were recovered as a result of action 5 of 13 TALENs tested. Moreover, a large deletion was detected when two TALENs with recognition sequences of more than 1.3 kb apart were co-delivered into rice protoplasts.

An alternative approach to the stable integration of the TALEN cassette was proposed by the Daniel Voytas Lab in 2014 [55]. Transient expression of sequence-specific nucleases in tobacco leaves using a geminivirus resulted in the recovery of NHEJ events at the target regions of the three nucleases tested (ZFN, TALEN, and CRIPSPR/Cas9). Moreover, the co-delivery of DNA repair templates using the *bean yellow dwarf* virus resulted in GT events at the rates from one to two orders of magnitude over the conventional *Agrobacterium tumefaciens* T-DNA delivery. Interestingly, the authors observed a low level of NHEJ events and a high frequency of GT in the cells. Based on the experiments, they speculated that the effect was caused by a combination of targeted DSBs, a high replication of a repair template and a pleiotropic effect of the *trans*-acting replication-initiation protein (Rep) and RepA. With this technique, it was possible to regenerate plants with a desired change in the DNA sequence in less than 6 weeks. The proposed approach holds a big promise for the genome editing in monocots because some of the geminiviruses belonging to a genus *Mastrevirus* (eg, wheat dwarf virus and maize streak virus) have been successfully used for protein expression in monocots [55].

3.2 The Application of TALENs in Crops

The utilization of TALENs for crop improvement were clearly shown in few reports [56–59]. The most prominent improvements were the disruption of two fatty desaturase genes (*FAD2-1A* and *FAD2-1B*) in soybean [56], targeted mutations of three homoeoalleles that encode the MILDEW-RESISTANCE LOCUS (MLO) proteins in wheat [57], the mutation at the promoter site of the barley phytase gene of the purple acid phosphatase group named *HvPAPhy_a* [58], and the disruption of a *PROCERA* (*PRO*) gene in tomato [59]. Simultaneous mutations in the *FAD2-1A* and *FAD2-1B* genes resulted in the generation of lines low in polyunsaturated fats that have an economic value for increasing oil shelf life and improving oxidative stability. After segregation, mutant plants were isolated that lacked the TALEN transgene and carried only the targeted mutations. Furthermore, a new trait not found in nature was developed using TALENs after simultaneous targeting of three homoalleles in wheat [57]. TALEN-induced disruption of all three *TaMLO* homologs in the same plant conferred heritable broad-spectrum resistance to powdery mildew.

An increase in the cold storage and processing of potato tubers was achieved by targeted disruption of a *VACUOLAR INVERTASE* gene (*VInv*) that encodes an enzyme involved in hydrolysis of sucrose to glucose and fructose [60]. Full *VInv*-knockout plants had undetectable levels of reducing sugars that can form a potential carcinogen when reacted with free

amino acids upon high-temperature processing. As in the case of the soybean study, the authors managed to select plants that did not contain TALEN transgenes in the genome but only mutations in *VInv* alleles. The edited potato is void of the regulation covering GMO crops in the USA and may soon enter a market as the first crop edited with designed endonucleases (http://www.aphis.usda.gov/biotechnology/downloads/reg_loi/aphis_response_cellectis_potato.pdf).

3.3 Potential Limitations of the TALEN Technology

The number of endogenous sequences that can be targeted by TALENs are limited by the need of a thymidine nt at the 5′ position [61]. Each TALEN must be experimentally validated since not all de novo assembled TALEN pairs work efficiently in vivo [62]. In addition, conventional TALENs are not able to cleave DNA containing 5-methylcytosine. Since methylated cytosine is indistinguishable from thymidine in the major groove, a repeat that recognizes cytosine can be substituted for one that binds to thymine. This approach, however, can reduce the target specificity [63,64].

The construction of multiple repeat sequences to assemble the DNA-binding domains remains a challenging task. The repetitive nature of TALE arrays makes it difficult to amplify them with PCR, and the assembled TALENs can be mutated by recombination in vivo [65]. Different methods have been developed to simplify the cloning of repeat arrays [49,51,66], and various computer programs are available for efficient design of TALEs and target prediction [67]. The most popular assembly method is a Golden Gate platform which offers a rapid, inexpensive and user-friendly protocol for TALEN assembly.

4. THE CRISPR/CAS9 SYSTEM FOR THE GENOME ENGINEERING OF PLANTS

An RNA-based and very efficient genome-editing tool was developed using the bacterial CRISPR and Cas9 protein. The CRISPR arrays were first identified in the *Escherichia coli* genome in 1987 [68], but their biological relevance was not known. In 2005, it was shown that some of the regions of the CRISPR sequence were homologous to viral and plasmid DNA, suggesting a role in adaptive immunity [69–71]. Later on, the CRISPR arrays were confirmed to provide protection against invading viruses when combined with the Cas genes, and the mechanism of this RNA-mediated DNA-targeting immune system was demonstrated [72–75].

Although the CRISPR/Cas system is present in most of the archaeal and many bacterial genomes [76], the most used CRISPR/Cas genome-editing tool originates from *Streptococcus pyogenes*. It contains the minimal CRISPR machinery composed of a single Cas9 protein, CRISPR RNA (crRNA) with a complementary sequence to the target site, and a *trans*-activating RNA (tracrRNA) that forms a hairpin with crRNA [41]. The CRISPR/Cas system is a part of an adaptive immune system that protects bacteria and archaea from viruses by digesting their DNA in a sequence-specific manner. The immunity is attained by the incorporation of short fragments of the viral DNA known as spacers at the proximal end of the CRISPR locus between two repeat arrays [77]. The CRISPR sequence is transcribed during subsequent infections with the virus and is sliced into 40 nt-long crRNAs. Eventually, crRNAs are combined with the tracrRNA to activate and guide the Cas9 nuclease to the invading DNA. The Cas9 enzyme cleaves the homologous DNA sequences into fragments called protospacers [72]. Binding specificity is provided by the so-called "seed sequence" of about 12 bases and a short DNA sequence termed a protospacer adjacent motif (PAM). The PAM usually contains a sequence of 5′-NGG-3′ (less frequently 5′-NAG-3′ [78]) and is situated downstream of the target DNA [79] (Fig. 12.4).

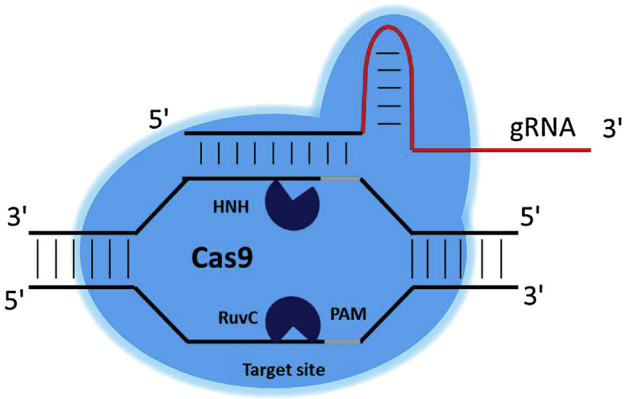

FIGURE 12.4 **The CRISPR/Cas system.** The system consists of a guided RNA (gRNA) and a Cas9 endonuclease. While gRNA is responsible for the specificity, the Cas9 protein mediates the cleavage of a complementary transcript. Cas9 requires the presence of a correct protospacer adjacent motif (PAM) sequence at the 3′ end of the target transcript, and upon binding to DNA, the nuclease unwinds the duplex and cleaves strands using the catalytic domains HNH and RuvC.

The progress for establishing the CRISPR/Cas system as a genome-editing tool was achieved when it was demonstrated that the target DNA sequence could be reprogrammed simply by replacing 20 nt in crRNA. In addition, crRNA could be combined with tracrRNA in a chimeric single-guide RNA (gRNA), thus reducing the system from three to two components and making it more efficient [80,81]. In comparison to the ZFN and TALEN technology, the CRISPR/Cas system relies on a simple Watson–Crick base pairing between gRNA and the target DNA sequence; therefore, the sophisticated protein engineering of each target is omitted [77]. The digestion of the target DNA sequence is performed by two cleavage domains (RuvC and HNH) of Cas9. The cleavage domains produce DSB at a position that is 3 nt upstream of PAM leaving in most of the cases blunt ends [80].

Another unique feature of the CRISPR/Cas system that sets it apart from other designed nucleases is the ability to selectively target either DNA or RNA. For instance, the Type III-B CRISPR/Cas system from *Pyrococcus furiosus* mediates the homology-dependent degradation of complementary RNA guided by an engineered crRNA [82]. The posttranscriptional control of gene expression would possibly be a more powerful alternative to RNA interference when the binding of the designed endonuclease to the target DNA is inhibited either by chromatin structure or by the presence of other bound proteins. In addition, the target elimination of only one of several splice variants from a single transcript could be possibly achieved. This is the gene expression regulation that is currently impossible to obtain by targeted DNA mutagenesis [77].

Everything that can be achieved with ZFNs and TALENs can in general be achieved with the CRISPR/Cas technology. The first publications on the utilization of the CRIPSR/Cas system in eukaryotes (human, mouse, and zebrafish) demonstrated that it is a simple, inexpensive, and versatile tool for genome editing [62,83–85].The target mutation efficiency of the CRISPR/Cas9 system in zebrafish embryos was found to be similar to that of ZFNs and TALENs [62]. The design and assembly of the CRISPR/Cas9 cassettes is relatively straightforward, currently devoid of intellectual property barriers, and thus can be preferred over other designed nucleases for genome-editing applications both in basic and applied studies [41].

4.1 The Application of the CRISPR/Cas System in Model Plant Species

In 2013, five reports demonstrated the use of the CRISPR/Cas9 system for gene disruption/targeting in *Arabidopsis*, tobacco, and rice [86–89]. All studies used a range of transformation platforms, including protoplast transformation, transient and stable *Agrobacterium*-mediated DNA delivery into leaves as rapid methods for the CRISPR/Cas9 system screening. A stable integration of the CRISPR/Cas cassette into the *Arabidopsis* and rice genomes resulted in the recovery of mutants with an expected phenotype already in the T1 generation of multiple genes that were targeted [86]. The mutation frequency was high in both *Arabidopsis* and rice, ranging in most of the cases from 26% to 84%. Similar to other designed endonucleases, multiple mutated alleles with different indels were recovered from transgenic plants, indicating DNA repair through the NHEJ pathway. The successful application of the CRISPR/Cas system for the targeted mutagenesis in monocots (rice and sorghum) and dicots (*Arabidopsis* and tobacco) was shown in a separate study [90]. Overall, when stably integrated, the CRIPSR/Cas technique can generate detectable mutations at a frequency of 50–89% for a single locus and 68–74% for double loci in plants [91]. In line with other designed endonucleases, it was possible to isolate transgene-free *Arabidopsis* plants with specific and heritable genome-editing events. In addition, the main practical advantage of CRISPR/Cas9 over ZFNs and TALENs is the ease of multiplexing. It simply requires the monomeric Cas9 protein and selected sequence-specific gRNAs [91]. On the other hand, multiplex editing with either ZFNs or TALENs demands separate dimeric proteins assembled for each target site [77]. The simultaneous introduction of targeted mutations at multiple sites can be used either to knock out redundant genes, parallel pathways or to create large genomic deletions/inversions [81,86,92]. More importantly, it has been noted that a high mutation frequency observed in rice (up to 91.6%) is apparently due to the unique feature of the CRISPR/Cas system (unlike ZFN and TALEN) to tolerate DNA methylation at cleavage sites [78,93]. This makes the CRISPR/Cas technology more favorable over other designed endonucleases because about 70% of the CG/CNG sites are methylated in plants [94]. The CRISPR/Cas9 system is therefore more useful for genome editing in plants, such as monocots with high genomic GC content [57,93].

In plants, gRNAs can be expressed under the control of different promoters that are recognized by RNA polymerase II and III, such as U6-26, *AtU6*, *OsU6*, *AtUBQ*, *OsUBQ*, and *CaMV 35S* [86,91,95]. Similarly, the expression of the Cas9 endonuclease can be driven by either *EF1A*, *CaMV*, *UBO*, or *LTR* promoters. Among them, the *CaMV* 35S promoter has been used most often [96] to drive the expression of a single chimeric gRNA that has been shown to be more efficient than separate crRNA and tracrRNA components for site-targeted mutagenesis in plants [81,93]. Although, due to the differences in experimental setups, it is hard to compare transformation and detection methods; but in general, the targeting efficiency of the CRISPR/Cas9 system seems to be comparable to or exceeding that obtained with ZFNs and TALENs [97,98].

One of the criticisms of the CRISPR/Cas technology is the relatively high rate of off-target effects reported in studies on animals [78,99,100]. Similarly, the off-target mutagenesis was observed in rice in two separate studies by using the PCR/restriction enzyme assay [88,89]. At the same time, no off-target modifications have been observed in studies on

Arabidopsis, tobacco, sweet orange, and in a separate study on rice using different methods, including sequencing of PCR amplicons, the whole-genome sequencing, and the restriction enzyme loss method [81,87,101,102]. The reduced specificity of the CRISPR/Cas system in some of the previous reports is apparently due to the fact that only a fragment of 8–12 nt at the 3′-end (the seed sequence) is needed for target site recognition and cleavage [90,103]. In addition, multiple mismatches in the PAM-distal region can be tolerated, depending on a sequence [78,99,100]. It has been hypothesized that the reduced specificity of the CRISPR/Cas9 complex at nonseed positions in the crRNA spacer has evolved to decrease the escape of viruses with point mutations form the immune system of bacteria [104].

Overall, the limited data available thus far suggest that the off-target effects caused by the CRISPR/Cas system are rare in plants. Nevertheless, a careful selection of the specific gRNA sequences combined with the proper regulation of the CRISPR/Cas cassette expression should reduce the risk of unwanted genome modifications.

4.2 The Application of the CRISPR/Cas System in Crops

A simplicity in both design and assembly and an open access to the components of the CRISPR/Cas system made it highly applicable for the range of crops, including rice, sorghum [90], wheat [57,92], maize [98], tomato [105], and sweet orange [106]. Curiously, four independent reports have shown that the CRISPR/Cas9 technology is suitable for the introduction of biallelic or homozygous mutations directly in the first generation of stable transgenic rice and tomato plants [81,88,107,108]. More importantly, genetic crosses segregating the CRISPR/Cas cassettes away from the edited plants have allowed to obtain genome-edited but transgene-free rice [81]. These studies indicate an exceptionally high efficiency of the CRISPR/Cas system in agriculturally important crop species.

In 2014, the CRISPR/Cas9 technology was briefly characterized for its mutation efficiency in one of the most complicated sequenced genomes—bread wheat (*Triticum aestivum*) [57]. A stable transformation of the CRISPR/Cas9 cassette resulted in the recovery of mature plants with mutations at one of the three alleles of the *MILDEW RESISTANCE LOCUS* (*MLO-A1*) gene with a frequency of 5.6% comparable to that obtained by TALENs [57]. Future reports will demonstrate how efficient the CRISPR/Cas9 technology is for targeted mutagenesis of all alleles simultaneously in hexaploid wheat. The possibility of a relatively easy multiplexing and tolerance of the Cas9 enzyme to DNA methylation leaves very little doubt to suspect that the CRISPR/Cas9 system would not be efficient in the most complicated crop genomes.

4.3 Potential Limitations of the CRISPR/Cas System

One of the biggest concerns regarding the CRISPR/Cas system is its relatively high off-target mutagenesis reported previously in animals [78,99]. However, this seems to be not of a big concern for plants, possibly due to differences in the transformation efficiency, expression levels, and codon usage in plant systems. The optimization of Cas9 nuclease expression has been proposed as a way to control the specificity because high concentrations of Cas9 and gRNA components can cause off-target effects in animals [78,100,109]. Another approach is to carefully select target regions in the genome because the imperfectly matched spacer sequences can result in the cleavage at off-target positions. A comparison of several gRNAs targeting the same gene in human cells has revealed that the CRISPR/Cas system is less efficient at the sequences with an unusually high or low GC content as compared to those with an average GC level [57]. In addition, gRNAs designed to target a transcribed strand are less effective than those targeting a nontranscribed strand. Furthermore, the Cas9 enzyme preferentially binds to gRNAs containing purine residues in the last four positions of spacer sequence with a direct correlation between the affinity of Cas9 to gRNA and the cleavage activity. Although it still remains to be shown whether the same rules are applicable to plant systems, these examples can be taken into account for gRNA design in plants in order to increase the efficiency and reduce off-target effects of the CRISPR/Cas technology.

Unfortunately, the possibility of the CRISPR/Cas9 system to target a desired sequence may be limited by the availability of PAM sites [80]. The alteration of the PAM sequence greatly reduces but not abolishes the activity of the CRISPR/Cas system in plants, suggesting that although PAM is important, it is not absolutely required for the function of CRISPR/Cas [91]. A thorough examination of nuclear genome sequences in silico from eight representative plant species (*Arabidopsis thaliana*, *Medicago truncatula*, *G. max*, *Solanum lycopersicum*, *Brachpodium distachyon*, *Oryza sativa*, *Sorghum bicolor*, and *Zea mays*) using data from mammalian systems has revealed an occurrence of the PAM (NGG/NAG) site at the frequency of 5–12 times for every 100 bp [110]. The total number of PAMs correlated with genome size, and for all species except maize, it was possible to predict specific gRNAs to target 85.4–98.9% of the annotated transcript units. Since maize has the largest genome examined and the functional redundancy of some homologous genes with high sequence identity, only 30% of the transcription units could be targeted by specific gRNAs. It is, therefore, expected that similar challenges may occur for gRNA target prediction in wheat and barley that have even larger genomes than maize [110].

5. FUTURE PERSPECTIVES OF THE GENOME-EDITING TECHNOLOGY

The use of the ZFN, TALEN, and CRISPR/Cas technologies to target DSBs to the selected locus/loci has opened up the possibility of a precise, fast, and efficient genome editing both in prokaryotes and eukaryotes. The utilization of designed endonucleases will accelerate both functional genomics and applied crop improvement [3]. The connection between a particular gene and the resulted phenotype would be easy to establish for species in which a mutant is unknown or does not exist in nature. This, in turn, should speed up the efforts for the development of novel traits. Eventually, the products obtained by site-specific nucleases which do not contain a transgene cassette are expected to be regulated in the North America similar to conventionally bred genotypes, and thus be more cost effective to bring to market (http://www.genengnews.com/insight-and-intelligence/gene-editing-will-change-everything-just-not-all-at-one-time/77900351/). The use of designed endonucleases may remove a number of regulatory restrictions associated with transgenic plants. Although, the European regulatory organizations working on GM crops focus on the method and not the product (eg, plants produced by conventional mutagenesis and genome editing would be regulated differently under the current guidelines), there is a possibility that plants altered by the targeted mutagenesis using designed endonucleases would not be classified and regulated as GMOs [111,112].

The targeted, predicted integration of transgenes through a trait stacking approach can eliminate the resulting effect of an unintended disruption of host metabolism and/or production of toxic or allergenic compounds. By trait stacking, the plant cells can be efficiently engineered to act as a factory for the production of specific metabolites or proteins with a number of genes involved. This can be achieved by a careful examination of different loci in the plant genome for the influence of the chromatin and surrounding sequence on transgene expression. Eventually, a generic recipient line with a predetermined and characterized locus can be established for routine utilization of transgene insertion and strong expression, thus producing a high yield of the corresponding product [77].

Overall, it is expected that the CRISPR/Cas technology will advance more rapidly as compared to ZFNs and TALENs [113]. This is due to a combination of the few major factors: a simplicity in design and construction, a possibility of relatively easy multiplex targeting, tolerance to DNA methylation, and, most importantly, the open access policy of the CRISPR research community. Plasmids are freely available from the nonprofit repository (eg, Addgene), and the range of web tools have been developed for selecting gRNA sequences and predicting their specificity (eg, CRISPR-P, CRISPR-PLANT, and Cas-OFFinder) [77].The application of this tool in plants opens immense possibilities from the regulation of lignin biosynthesis in order to increase forage digestibility and kappa value in the pulping industry [44] to the generation of wheat-resistant cultivars by targeting the loss of susceptibility genes [114]. For these targets to be met in plants, the development of supporting technologies is required. In most of the cases, the limiting factor is the availability of an efficient transformation technique and a high-throughput molecular screening method for genome-editing analysis. Therefore, the improvement in cell and tissue culture together with the development of more efficient transformation techniques will continue to play an essential role in the further development of genome-editing technology in plants [3]. One of the promising approaches includes the utilization of a microspore culture together with the protein-mediated genome editing [115]. Overall, a brief overview of examples of targeted genome modification in plants mediated by designed endonucleases provides a clear indication that complex crop genomes can now be manipulated with a precision that far surpasses the conventional breeding practices. Therefore, it can be speculated that it is just a matter of time when genome-edited fruit, vegetable, and cereal crops will appear on the shelves of stores.

GLOSSARY

Clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 An RNA-based genome-editing tool that consists of *Streptococcus pyogenes* Cas9 endonuclease and guided RNA.

Engineered endonucleases Artificial endonucleases that can be designed to digest a predetermined nucleotide sequence.

Genome editing A type of genome manipulation for the purpose of insertion, deletion, or replacement of the DNA sequence by using engineered endonucleases.

Homologous recombination repair A type of double-strand break repair in which the nucleotide sequences are exchanged between two identical or very similar molecules of DNA during genetic recombination.

Homing endonucleases/meganucleases Naturally occurring endonucleases characterized by a high specificity due to a long recognition site (12–40 bp).

Nonhomologous end joining repair A double-strand break repair pathway that involves a simple rejoining of the broken ends of the DNA molecule either in the presence or absence of the microhomology regions between broken ends.

Transcription activator-like effector nucleases The engineered endonucleases that contain a TALE DNA-binding domain at the amino terminus and a *Fok*I nuclease domain at the carboxyl terminus.

Zinc-finger nucleases The engineered endonucleases that consist of a zinc-finger protein at the N-terminus and a *Fok*I nuclease domain at the C-terminus.

LIST OF ACRONYMS AND ABBREVIATIONS

ABI4 An *ABAINSENSITIVE-4* gene
ADH1 An *ALCOHOL DEHYDROGENASE-1* gene
ALS An *ACETOLACTATE SYNTHASE* gene
CLV3 A *CLAVATA3* gene
crRNA CRISPR RNA
CRISPR/Cas Clustered regularly interspaced short palindromic repeats/CRISPR-associated
DCL1a A *DICER-LIKE1a* gene
DSB Double-strand break
EMN Engineered homing endonucleases/meganucleases
ES Embryonic stem cells
FAD2-1A* and *FAD2-1B Fatty desaturase genes
GFP GREEN FLUORESCENT PROTEIN
GMO Genetically modified organisms
gRNA Single guide RNA
GT Gene targeting
HEN1a *HUA ENHANCER 1a*gene
HR Homologous recombination
Indel Mutation caused either by insertion or deletion
IPK1 Gene that encodes the inositol-1,3,4,5,6-pentakisphosphate 2-kinase gene
MLO MILDEW-RESISTANCE LOCUS
NHEJ Nonhomologous end joining
NLS Nuclear localization signal
PAM Protospacer adjacent motif
PCR Polymerase chain reaction
PPOX *PROTOPORPHYRINOGEN OXIDASE* gene
PRO *PROCERA* gene
RDR6a *RNA-DEPENDENT RNA POLYMERASE 6a* gene
Rep Replication-initiation protein
RVD Repeat variable diresidue
SuRA* and *SuRB *SULFONYLUREA RECEPTOR* gene
SSA Single-strand annealing
TALEN Transcription activator-like effector nucleases
TLPs Trait landing pads
tracrRNA *Trans*-activating RNA
TT4 *TRANSPARENT TESTA-4* gene
VInv *VACUOLAR INVERTASE* gene
YFP Yellow fluorescent protein
ZFN Zinc-finger nucleases

REFERENCES

[1] Stadler LJ. Genetic effects of X-Rays in maize. Proc Natl Acad Sci USA 1928;14(1):69–75.
[2] Stadler LJ. Mutations in barley induced by X-Rays and radium. Science 1928;68(1756):186–7.
[3] Petolino JF. Genome editing in plants via designed zinc finger nucleases. In Vitro Cell Dev Biol Plant 2015;51(1):1–8.
[4] Bibikova M, Beumer K, Trautman JK, Carroll D. Enhancing gene targeting with designed zinc finger nucleases. Science 2003;300(5620):764.
[5] Puchta H. Gene replacement by homologous recombination in plants. Plant Mol Biol 2002;48(1–2):173–82.
[6] Sakuma T, Woltjen K. Nuclease-mediated genome editing: at the front-line of functional genomics technology. Dev Growth Differ 2014;56(1):2–13.
[7] Zhang Y, Zhang F, Li X, Baller JA, Qi Y, Starker CG, et al. Transcription activator-like effector nucleases enable efficient plant genome engineering. Plant Physiol 2013;161(1):20–7.
[8] Terada R, Urawa H, Inagaki Y, Tsugane K, Iida S. Efficient gene targeting by homologous recombination in rice. Nat Biotechnol 2002;20(10):1030–4.
[9] Hanin M, Volrath S, Bogucki A, Briker M, Ward E, Paszkowski J. Gene targeting in *Arabidopsis*. Plant J 2001;28(6):671–7.
[10] Choulika A, Perrin A, Dujon B, Nicolas JF. Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of *Saccharomyces cerevisiae*. Mol Cell Biol 1995;15(4):1968–73.
[11] Cohen-Tannoudji M, Robine S, Choulika A, Pinto D, El Marjou F, Babinet C, et al. I-SceI-induced gene replacement at a natural locus in embryonic stem cells. Mol Cell Biol 1998;18(3):1444–8.

[12] Puchta H, Dujon B, Hohn B. Homologous recombination in plant cells is enhanced by in vivo induction of double strand breaks into DNA by a site-specific endonuclease. Nucleic Acids Res 1993;21(22):5034–40.
[13] Puchta H, Dujon B, Hohn B. Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination. Proc Natl Acad Sci USA 1996;93(10):5055–60.
[14] Gorbunova V, Levy AA. Non-homologous DNA end joining in plant cells is associated with deletions and filler DNA insertions. Nucleic Acids Res 1997;25(22):4650–7.
[15] Nakatsukasa T, Shiraishi Y, Negi S, Imanishi M, Futaki S, Sugiura Y. Site-specific DNA cleavage by artificial zinc finger-type nuclease with cerium-binding peptide. Biochem Biophys Res Commun 2005;330(1):247–52.
[16] Mahfouz MM, Piatek A, Stewart Jr CN. Genome engineering via TALENs and CRISPR/Cas9 systems: challenges and perspectives. Plant Biotechnol J 2014;12(8):1006–14.
[17] Osakabe Y, Osakabe K. Genome editing with engineered nucleases in plants. Plant Cell Physiol 2015;56(3):389–400.
[18] Gaj T, Gersbach CA, Barbas 3rd CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol 2013;31(7):397–405.
[19] Enuameh MS, Asriyan Y, Richards A, Christensen RG, Hall VL, Kazemian M, et al. Global analysis of *Drosophila* Cys(2)-His(2) zinc finger proteins reveals a multitude of novel recognition motifs and binding determinants. Genome Res 2013;23(6):928–40.
[20] Persikov AV, Wetzel JL, Rowland EF, Oakes BL, Xu DJ, Singh M, et al. A systematic survey of the Cys2His2 zinc finger DNA-binding landscape. Nucleic Acids Res 2015;43(3):1965–84.
[21] Kim Y-G, Shi Y, Berg JM, Chandrasegaran S. Site-specific cleavage of DNA–RNA hybrids by zinc finger/FokI cleavage domain fusions. Gene 1997;203(1):43–9.
[22] Bitinaite J, Wah DA, Aggarwal AK, Schildkraut I. FokI dimerization is required for DNA cleavage. Proc Natl Acad Sci 1998;95(18):10570–5.
[23] Orlando SJ, Santiago Y, DeKelver RC, Freyvert Y, Boydston EA, Moehle EA, et al. Zinc-finger nuclease-driven targeted integration into mammalian genomes using donors with limited chromosomal homology. Nucleic Acids Res 2010;38(15):e152.
[24] Bibikova M, Golic M, Golic KG, Carroll D. Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. Genetics 2002;161(3):1169–75.
[25] Shukla VK, Doyon Y, Miller JC, DeKelver RC, Moehle EA, Worden SE, et al. Precise genome modification in the crop species Zea mays using zinc-finger nucleases. Nature 2009;459(7245):437–41.
[26] Townsend JA, Wright DA, Winfrey RJ, Fu F, Maeder ML, Joung JK, et al. High-frequency modification of plant genes using engineered zinc-finger nucleases. Nature 2009;459(7245):442–5.
[27] Osakabe K, Osakabe Y, Toki S. Site-directed mutagenesis in *Arabidopsis* using custom-designed zinc finger nucleases. Proc Natl Acad Sci 2010;107(26):12034–9.
[28] Zhang F, Maeder ML, Unger-Wallace E, Hoshaw JP, Reyon D, Christian M, et al. High frequency targeted mutagenesis in *Arabidopsis thaliana* using zinc finger nucleases. Proc Natl Acad Sci 2010;107(26):12028–33.
[29] Curtin SJ, Zhang F, Sander JD, Haun WJ, Starker C, Baltes NJ, et al. Targeted mutagenesis of duplicated genes in soybean with zinc-finger nucleases. Plant Physiol 2011;156(2):466–73.
[30] Lloyd A, Plaisier CL, Carroll D, Drews GN. Targeted mutagenesis using zinc-finger nucleases in *Arabidopsis*. Proc Natl Acad Sci USA 2005;102(6):2232–7.
[31] De Pater S, Neuteboom LW, Pinas JE, Hooykaas PJJ, Van Der Zaal BJ. ZFN-induced mutagenesis and gene-targeting in Arabidopsis through Agrobacterium-mediated floral dip transformation. Plant Biotechnol J 2009;7(8):821–35.
[32] Tovkach A, Zeevi V, Tzfira T. A toolbox and procedural notes for characterizing novel zinc finger nucleases for genome editing in plant cells. Plant J 2009;57(4):747–57.
[33] Marton I, Zuker A, Shklarman E, Zeevi V, Tovkach A, Roffe S, et al. Nontransgenic genome modification in plant cells. Plant Physiol 2010;154(3):1079–87.
[34] Cai C, Doyon Y, Ainley WM, Miller J, DeKelver R, Moehle E, et al. Targeted transgene integration in plant cells using designed zinc finger nucleases. Plant Mol Biol 2009;69(6):699–709.
[35] Petolino J, Worden A, Curlee K, Connell J, Strange Moynahan T, Larsen C, et al. Zinc finger nuclease-mediated transgene deletion. Plant Mol Biol 2010;73(6):617–28.
[36] Qi Y, Li X, Zhang Y, Starker CG, Baltes NJ, Zhang F, et al. Targeted deletion and inversion of tandemly arrayed genes in *Arabidopsis thaliana* using zinc finger nucleases. G3 Genes|Genomes|Genetics 2013;3(10):1707–15.
[37] Haber JF, Partners and pathways. Trends Genet 2000;16(6):259–64.
[38] Pater S, Pinas JE, Hooykaas PJJ, Zaal BJ. ZFN-mediated gene targeting of the *Arabidopsis* protoporphyrinogen oxidase gene through Agrobacterium-mediated floral dip transformation. Plant Biotechnol J 2013;11(4):510–5.
[39] Petolino JF, Hopkins NL, Kosegi BD, Skokut M. Whisker-mediated transformation of embryogenic callus of maize. Plant Cell Rep 2000;19(8):781–6.
[40] Ainley WM, Sastry-Dent L, Welter ME, Murray MG, Zeitler B, Amora R, et al. Trait stacking via targeted genome editing. Plant Biotechnol J 2013;11(9):1126–34.
[41] Kumar V, Jain M. The CRISPR-Cas system for plant genome editing: advances and opportunities. J Exp Bot 2015;66(1):47–57.
[42] Sander JD, Dahlborg EJ, Goodwin MJ, Cade L, Zhang F, Cifuentes D, et al. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). Nat Methods 2011;8(1):67–9.

[43] Chandrasekharan S, Kumar S, Valley CM, Rai A. Proprietary science, open science and the role of patent disclosure: the case of zinc-finger proteins. Nat Biotechnol 2009;27(2):140–4.
[44] Eudes A, Liang Y, Mitra P, Loque D. Lignin bioengineering. Curr Opin Biotechnol 2014;26:189–98.
[45] Christian M, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A, et al. Targeting DNA double-strand breaks with TAL effector nucleases. Genetics 2010;186(2):757–61.
[46] Bogdanove AJ, Schornack S, Lahaye T. TAL effectors: finding plant genes for disease and defense. Curr Opin Plant Biol 2010;13(4):394–401.
[47] Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, et al. Breaking the code of DNA binding specificity of TAL-type III effectors. Science 2009;326(5959):1509–12.
[48] Moscou MJ, Bogdanove AJ. A simple cipher governs DNA recognition by TAL effectors. Science 2009;326(5959):1501.
[49] Weber E, Gruetzner R, Werner S, Engler C, Marillonnet S. Assembly of designer TAL effectors by Golden Gate cloning. PLoS One 2011;6(5):e19722.
[50] Mahfouz MM, Li L, Shamimuzzaman M, Wibowo A, Fang X, Zhu J-K. De novo-engineered transcription activator-like effector (TALE) hybrid nuclease with novel DNA binding specificity creates double-strand breaks. Proc Natl Acad Sci 2011;108(6):2623–8.
[51] Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C, et al. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res 2011;39(12):e82.
[52] Christian M, Qi Y, Zhang Y, Voytas DF. Targeted mutagenesis of *Arabidopsis thaliana* using engineered TAL effector nucleases. G3 (Bethesda) 2013;3(10):1697–705.
[53] Forner J, Pfeiffer A, Langenecker T, Manavella P, Lohmann JU. Germline-transmitted genome editing in *Arabidopsis thaliana* Using TAL-effector-nucleases. PLoS One 2015;10(3):e0121056.
[54] Shan Q, Wang Y, Chen K, Liang Z, Li J, Zhang Y, et al. Rapid and efficient gene modification in rice and *Brachypodium* using TALENs. Mol Plant.6(4):1365–1368.
[55] Baltes NJ, Gil-Humanes J, Cermak T, Atkins PA, Voytas DF. DNA replicons for plant genome engineering. Plant Cell 2014;26(1):151–63.
[56] Haun W, Coffman A, Clasen BM, Demorest ZL, Lowy A, Ray E, et al. Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. Plant Biotechnol J 2014;12(7):934–40.
[57] Wang Y, Cheng X, Shan Q, Zhang Y, Liu J, Gao C, et al. Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat Biotechnol 2014;32(9):947–51.
[58] Wendt T, Holm P, Starker C, Christian M, Voytas D, Brinch-Pedersen H, et al. TAL effector nucleases induce mutations at a pre-selected location in the genome of primary barley transformants. Plant Mol Biol 2013;83(3):279–85.
[59] Lor VS, Starker CG, Voytas DF, Weiss D, Olszewski NE. Targeted mutagenesis of the tomato PROCERA gene using transcription activator-like effector nucleases. Plant Physiol 2014;166(3):1288–91.
[60] Clasen BM, Stoddard TJ, Luo S, Demorest ZL, Li J, Cedrone F, et al. Improving cold storage and processing traits in potato through targeted gene knockout. Plant Biotechnol J 2015. n/a-n/a.
[61] Doyle EL, Booher NJ, Standage DS, Voytas DF, Brendel VP, Vandyk JK, et al. TAL effector-nucleotide targeter (TALE-NT) 2.0: tools for TAL effector design and target prediction. Nucleic Acids Res 2012;40(Web Server issue):W117–22.
[62] Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, et al. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotech 2013;31(3):227–9.
[63] Deng D, Yin P, Yan C, Pan X, Gong X, Qi S, et al. Recognition of methylated DNA by TAL effectors. Cell Res 2012;22(10):1502–4.
[64] Valton J, Dupuy A, Daboussi F, Thomas S, Marechal A, Macmaster R, et al. Overcoming transcription activator-like effector (TALE) DNA binding domain sensitivity to cytosine methylation. J Biol Chem 2012;287(46):38427–32.
[65] Holkers M, Maggio I, Liu J, Janssen JM, Miselli F, Mussolino C, et al. Differential integrity of TALE nuclease genes following adenoviral and lentiviral vector gene transfer into human cells. Nucleic Acids Res 2013;41(5):e63.
[66] Reyon D, Tsai SQ, Khayter C, Foden JA, Sander JD, Joung JK. FLASH assembly of TALENs for high-throughput genome editing. Nat Biotechnol 2012;30(5):460–5.
[67] Cermak T, Starker CG, Voytas DF. Efficient design and assembly of custom TALENs using the Golden Gate platform. Methods Mol Biol 2015;1239:133–59.
[68] Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. J Bacteriol 1987;169(12):5429–33.
[69] Bolotin A, Quinquis B, Sorokin A, Ehrlich SD. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology 2005;151(8):2551–61.
[70] Mojica FJ, Diez-Villasenor C, Garcia-Martinez J, Soria E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J Mol Evol 2005;60(2):174–82.
[71] Pourcel C, Salvignol G, Vergnaud G. CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. Microbiology 2005;151(3):653–63.
[72] Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, et al. CRISPR provides acquired resistance against viruses in prokaryotes. Science 2007;315(5819):1709–12.
[73] Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, et al. Small CRISPR RNAs guide antiviral defense in prokaryotes. Science 2008;321(5891):960–4.
[74] Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, et al. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 2011;471(7340):602–7.

[75] Garneau JE, Dupuis ME, Villion M, Romero DA, Barrangou R, Boyaval P, et al. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature 2010;468(7320):67–71.
[76] Rousseau C, Gonnet M, Le Romancer M, Nicolas J. CRISPI: a CRISPR interactive database. Bioinformatics 2009;25(24):3317–8.
[77] Bortesi L, Fischer R. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol Adv 2015;33(1):41–52.
[78] Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, et al. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol 2013;31(9):827–32.
[79] Qi Lei S, Larson Matthew H, Gilbert Luke A, Doudna Jennifer A, Weissman Jonathan S, Arkin Adam P, et al. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 2013;152(5):1173–83.
[80] Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 2012;337(6096):816–21.
[81] Zhou H, Liu B, Weeks DP, Spalding MH, Yang B. Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. Nucleic Acids Res 2014;42(17):10903–14.
[82] Hale CR, Zhao P, Olson S, Duff MO, Graveley BR, Wells L, et al. RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. Cell 2009;139(5):945–56.
[83] Cho SW, Kim S, Kim JM, Kim JS. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat Biotechnol 2013;31(3):230–2.
[84] Jinek M, East A, Cheng A, Lin S, Ma E, Doudna J. RNA-programmed genome editing in human cells. Elife 2013;2:e00471.
[85] Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, et al. RNA-guided human genome engineering via Cas9. Science 2013;339(6121):823–6.
[86] Feng Z, Zhang B, Ding W, Liu X, Yang D-L, Wei P, et al. Efficient genome editing in plants using a CRISPR/Cas system. Cell Res 2013;23(10):1229–32.
[87] Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S. Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. Nat Biotechnol 2013;31(8):691–3.
[88] Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, et al. Targeted genome modification of crop plants using a CRISPR-Cas system. Nat Biotech 2013;31(8):686–8.
[89] Xie K, Yang Y. RNA-guided genome editing in plants using a CRISPR–Cas system. Mol Plant 2013;6(6):1975–83.
[90] Jiang W, Zhou H, Bi H, Fromm M, Yang B, Weeks DP. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in *Arabidopsis*, tobacco, sorghum and rice. Nucleic Acids Res 2013;41(20):e188.
[91] Mao Y, Zhang H, Xu N, Zhang B, Gou F, Zhu JK. Application of the CRISPR-Cas system for efficient genome engineering in plants. Mol Plant 2013;6(6):2008–11.
[92] Upadhyay SK, Kumar J, Alok A, Tuli R. RNA-guided genome editing for target gene mutations in wheat. G3 (Bethesda) 2013;3(12):2233–8.
[93] Miao J, Guo D, Zhang J, Huang Q, Qin G, Zhang X, et al. Targeted mutagenesis in rice using CRISPR-Cas system. Cell Res 2013;23(10):1233–6.
[94] Vanyushin BF, Ashapkin VV. DNA methylation in higher plants: past, present and future. Biochim Biophys Acta 2011;1809(8):360–8.
[95] Fauser F, Schiml S, Puchta H. Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*. Plant J 2014;79(2):348–59.
[96] Belhaj K, Chaparro-Garcia A, Kamoun S, Nekrasov V. Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. Plant Methods 2013;9(1):39.
[97] Lozano-Juste J, Cutler SR. Plant genome engineering in full bloom. Trends Plant Sci 2014;19(5):284–7.
[98] Liang Z, Zhang K, Chen K, Gao C. Targeted mutagenesis in Zea mays using TALENs and the CRISPR/Cas system. J Genet Genomics 2014;41(2):63–8.
[99] Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat Biotechnol 2013;31(9):822–6.
[100] Pattanayak V, Lin S, Guilinger JP, Ma E, Doudna JA, Liu DR. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. Nat Biotechnol 2013;31(9):839–43.
[101] Feng Z, Mao Y, Xu N, Zhang B, Wei P, Yang DL, et al. Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in *Arabidopsis*. Proc Natl Acad Sci USA 2014;111(12):4632–7.
[102] Jiang W, Yang B, Weeks DP. Efficient CRISPR/Cas9-mediated gene editing in *Arabidopsis thaliana* and inheritance of modified genes in the T2 and T3 generations. PLoS One 2014;9(6):e99225.
[103] Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex genome engineering using CRISPR/Cas systems. Science 2013;339(6121):819–23.
[104] Semenova E, Jore MM, Datsenko KA, Semenova A, Westra ER, Wanner B, et al. Interference by clustered regularly interspaced short palindromic repeat (CRISPR) RNA is governed by a seed sequence. Proc Natl Acad Sci USA 2011;108(25):10098–103.
[105] Ron M, Kajala K, Pauluzzi G, Wang D, Reynoso MA, Zumstein K, et al. Hairy root transformation using *Agrobacterium rhizogenes* as a tool for exploring cell type-specific gene expression and function using tomato as a model. Plant Physiol 2014;166(2):455–69.
[106] Jia H, Wang N. Targeted genome editing of sweet orange using Cas9/sgRNA. PLoS One 2014;9(4):e93806.
[107] Brooks C, Nekrasov V, Lippman ZB, Van Eck J. Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. Plant Physiol 2014;166(3):1292–7.
[108] Zhang H, Zhang J, Wei P, Zhang B, Gou F, Feng Z, et al. The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. Plant Biotechnol J 2014;12(6):797–807.
[109] Fujii W, Kawasaki K, Sugiura K, Naito K. Efficient generation of large-scale genome-modified mice using gRNA and CAS9 endonuclease. Nucleic Acids Res 2013;41(20):e187.

[110] Xie K, Zhang J, Yang Y. Genome-wide prediction of highly specific guide RNA spacers for CRISPR–Cas9-mediated genome editing in model plants and major crops. Mol Plant 2014;7(5):923–6.
[111] Hartung F, Schiemann J. Precise plant breeding using new genome editing techniques: opportunities, safety and regulation in the EU. Plant J 2014;78(5):742–52.
[112] Podevin N, Davies HV, Hartung F, Nogue F, Casacuberta JM. Site-directed nucleases: a paradigm shift in predictable, knowledge-based plant breeding. Trends Biotechnol 2013;31(6):375–83.
[113] Belhaj K, Chaparro-Garcia A, Kamoun S, Patron NJ, Nekrasov V. Editing plant genomes with CRISPR/Cas9. Curr Opin Biotechnol 2015;32: 76–84.
[114] O'Driscoll A, Kildea S, Doohan F, Spink J, Mullins E. The wheat-*Septoria* conflict: a new front opening up? Trends Plant Sci 2014;19(9):602–10.
[115] Bilichak A, Luu J, Eudes F. Intracellular delivery of fluorescent protein into viable wheat microspores using cationic peptides. Front Plant Sci 2015;6:666.