

Directed genome engineering for genome optimization

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ABSTRACT The ability to develop nucleases with tailor-made activities for targeted DNA double-strand break induction at will at any desired position in the genome has been a major breakthrough to make targeted genome optimization feasible in plants. The development of site specific nucleases for precise genome modification has expanded the repertoire of tools for the development and optimization of traits, already including mutation breeding, molecular breeding and transgenesis. Through directed genome engineering technology, the huge amount of information provided by genomics and systems biology can now more effectively be used for the creation of plants with improved or new traits, and for the dissection of gene functions. Although still in an early phase of deployment, its utility has been demonstrated for engineering disease resistance, herbicide tolerance, altered metabolite profiles, and for molecular trait stacking to allow linked transmission of transgenes. In this article, we will briefly review the different approaches for directed genome engineering with the emphasis on double strand break (DSB)-mediated engineering towards genome optimization for crop improvement and towards the acceleration of functional genomics.

KEY WORDS: *DNA double strand break induction and repair, site-specific nuclease, genome editing, crop improvement*

Introduction

During the past 100 years, the yield of agricultural crops has steadily increased. For generations, crop plants have been genetically improved using conventional breeding methods and spontaneous or induced mutagenesis. Plant biotechnology took off 30 years ago with the ability to create transgenic plants through *Agrobacterium*-mediated transformation. Transgenesis dramatically increased the possibilities for crop improvement by expanding the pool of traits that could be integrated into the plant genome. It broke the reliance of breeders on existing biodiversity or on the genes of close relatives of a particular crop for additional genetic diversity. Transformation technology allows for the introduction of exogenous genes including such that do not exist naturally in the target crop. However, conventional mutation breeding and transformation are non-specific: mutation and transgene insertion happen at random positions. As a consequence, they require a screening step for selection of the desired mutants, or for selecting transformants with a preferred performance.

Through the modern era of systems biology and genomics, a huge amount of information is becoming available which enhances our understanding on trait-related genes and variants, on gene functions and on fundamental development, and on physiological and molecular processes. This refined understanding creates new opportunities for crop improvement and trait development. Although

breeding and transformation have resulted in major achievements, these not always allow to fully capture all opportunities arising from the fast developments in systems biology and genomics. Precise modification of plant genomes could enable to exploit more effectively the ever-increasing knowledge for purposes of both gene discovery and crop improvement. It would not only allow the targeted modifications in native genes, but also the introduction of foreign DNA into a predetermined location, the removal of undesired sequences or the optimization of a particular gene's sequence or its regulatory elements (Fig. 1).

Until recently, plants were recalcitrant to directed genome engineering. Induction of a targeted DNA double-strand break (DSB) by rare-cutting endonucleases such as I-SceI or I-CeuI has been shown to increase several fold the gene targeting frequency in tobacco and maize (Puchta *et al.*, 1996; Chilton and Que, 2003; D'Halluin *et al.*, 2008). However, the enhancement of gene targeting by these natural meganucleases was limited to loci with pre-engineered nuclease recognition sites. More recent advances in the field of custom-designed nucleases for the induction of a double strand break at a pre-determined position in the genome,

Abbreviations used in this paper: Cas, CRISPR-associated (endonuclease); DSB, DNA double-strand break; HR, homologous recombination; NHR, non-homologous recombination; TALEN, transcription activator-like effector nuclease; ZFN, zinc finger nuclease.

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have made plant directed genome engineering feasible.

Directed genome engineering through the use of site-specific nucleases for targeted DSB induction is based on the repair of the DSB by the plant's naturally occurring DNA repair pathways. Repair of the DSB by non-homologous recombination (NHR) will result mostly in non-precise repair with small insertions or deletions at the DSB site, a technology which is exploited for gene function discovery. Precise genome editing is possible through homologous recombination (HR)-mediated repair of the DSB. This requires the simultaneous introduction into the plant cell of the nuclease and the repair DNA that contains both the desired sequence modification and sequences homologous to those flanking the DSB site. The donor DNA, acting as a template for repair of the DSB, directs the modification of the chromosomal locus towards the desired changes (Fig. 1). Some excellent review articles on the use of designer nucleases for directed genome engineering in both model and crop plant species have been published (Tzifira *et al.*, 2012, Curtin *et al.*, 2012).

To create a DSB at a desired position in the genome, nowadays different tools are available and for all of them applications of agricultural relevance have been demonstrated. The zinc finger nucleases (ZFNs), consisting of a synthetic C2H2 zinc finger DNA-binding domain fused to the catalytic domain of the FokI endonuclease (Kim *et al.*, 1996), have been successfully used for directed genome engineering in plants (Wright *et al.*, 2005, Townsend *et al.*, 2009, Shukla *et al.*, 2009, De Pater *et al.*, 2013). By the use of ZFNs, it has been possible to introduce specific mutations in the acetolactate synthase genes (*SurA* and *SurB*) conferring herbicide resistance in tobacco (Townsend *et al.*, 2009). Shukla *et al.*, (2009) used ZFNs to precisely target a herbicide tolerance gene into the IPK1 locus of maize resulting in both herbicide resistance and reduced seed phytate content by targeted disruption of the IPK1 gene, a gene involved in the phytate biosynthesis pathway.

The transcription activator-like effector nucleases (TALENs), consisting of a TALE DNA-binding domain fused to the cleavage domain of the FokI endonuclease, are a rapidly emerging alternative for ZFNs as TALENs are easier to design (Cermak *et al.*, 2011) and do have less constraints in their DNA binding ability (Bogdanove *et al.*, 2010). TALENs have been successfully used in plants to generate disease resistance by targeted disruption of the rice bacterial blight susceptibility gene (Li *et al.*, 2012).

The LAGLIDADG homing endonucleases, also called meganucleases, are a third class of nucleases for targeted DSB induction (Epinat, 2003). Meganucleases are considerably more challenging to re-engineer compared to TALENs and ZFNs (Taylor *et al.*, 2012). The I-CreI homing endonuclease of *Chlamydomonas reinhardtii* (Thompson *et al.*, 1992) has been successfully re-engineered for targeted mutagenesis by NHR in the ligueless locus in maize (Gao *et al.*, 2010) and for targeted molecular trait stacking by HR in cotton (D'Halluin *et al.*, 2013).

More recently, a new approach for RNA-guided genome editing was described based on the CRISPR-associated (Cas) endonuclease. In contrast to the previous designer nucleases, ZFNs, TALENs and meganucleases, the RNA-guided Cas endonuclease does not each time require protein re-engineering to target a new sequence; only a new RNA guide is required and not a new enzyme. Recent papers have already shown the feasibility of RNA-programmed Cas9-driven genome editing in human cells, zebrafish and plants (Jinek *et al.*, 2013; Cong *et al.*, 2013; Mali *et al.*, 2013; Cho *et al.*, 2013; Hwang *et al.*, 2013; Shan *et al.*, 2013; Li *et al.*, 2013; Nekrasov *et al.*, 2013; Jiang *et al.*, 2013).

Directed genome engineering for trait stacking

To date, herbicide tolerance and/or insect resistance are the most widely used GM traits in crop plants. To cover for broad-spectrum combined weed and insect control, crops have to be engineered with multiple herbicide tolerance and insect resistance genes, with different modes of action. Beyond management for weed and insect control, there is a next wave of traits being developed to increase yield, quality, abiotic stress tolerance and disease resistance. Combining a large number of transgenic trait loci in one variety will become unmanageable through conventional plant breeding, as the size of the breeding populations increases exponentially with the number of trait loci to be combined. This requires the need to develop more efficient gene stacking technology to facilitate the combination of multiple trait transgenes into a single, genetic unit and as such to allow rapid introgression into commercially relevant germplasm.

Molecular trait stacks could be generated by transformation with a single construct carrying multiple trait genes, by co-delivery of single gene constructs or by re-transformation of an existing event with an additional construct. In all these cases, the traits have to be expressed as desired and have to be inherited as a single genetic locus. For molecular trait stacks generated via transformation with a multigene construct,

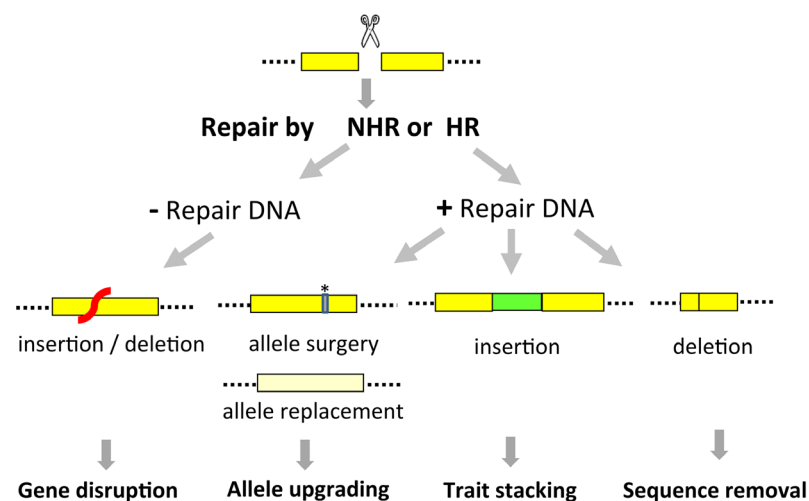
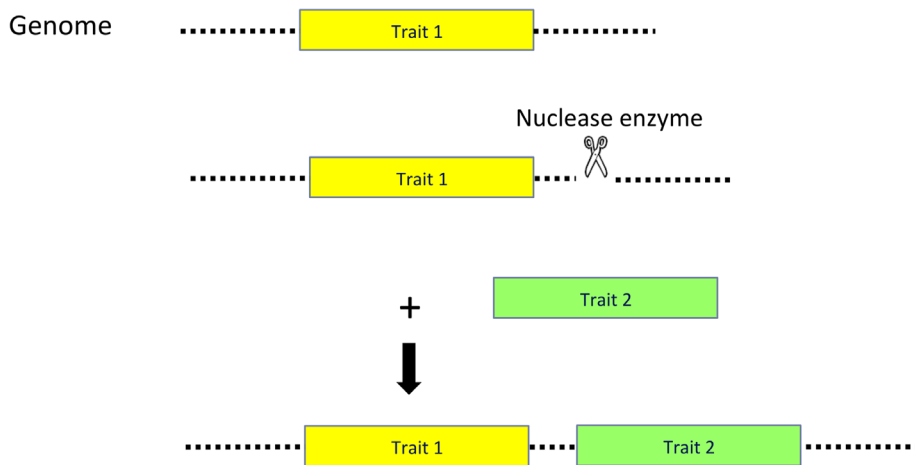


Fig. 1. Double strand break (DSB)-mediated genome engineering. A DSB is induced by a sequence-specific nuclease (scissor) in the target sequence (yellow box). The break can be repaired by non-homologous recombination (NHR) or homologous recombination (HR). Repair of the DSB by NHR is non-precise and can introduce insertions/deletions (denoted by the red region), resulting in gene disruption. Repair of the DSB by HR in the presence of a repair DNA can be very precise. The repair DNA represents the sequence modifications to be incorporated in the chromosomal locus: a single or a few basepair substitution (allele surgery *) or a larger sequence replacement (allele replacement) for allele upgrading; insertion (green box) for trait stacking; and deletion for removal of sequences.



e.g. Herbicide tolerance and Insect Resistance

Fig. 2. Targeted molecular trait stacking. A double strand break (DSB) is induced by a sequence-specific nuclease (scissor) in the flanking genomic sequence (dotted line) of an already existing transgene locus (Trait 1; yellow box). The DSB is repaired by targeted introduction of an additional trait gene (Trait 2; green box).

the technical challenge to identify an event with all stacked trait genes expressed as desired will increase with the number of trait genes included in the transformation vector, especially if stacked traits require very different promoters with different spatial and/or temporal specificities (Que *et al.*, 2010). In plants, enhancers present in promoters are able to change the activity of nearby promoters, resulting in loss of promoter specificity (Gudynaite-Savitch, 2009). Tandemly, divergently or convergently arranged genes may influence each other's expression due to transcriptional interference between these genes (Eszterhas *et al.*, 2002). In the cases of co-delivery of single gene constructs or re-transformation, an additional challenge is to select for plants with co-integrations that do not segregate in the following generations.

Targeted molecular trait stacking through targeted DSB induction could overcome some of the challenges associated with breeding stacks or molecular stacks. Through targeted molecular trait stacking, additional genes could be introduced in close vicinity of an already existing transgenic locus, within a genetic distance that would allow linked transmission, while the integration position

could be chosen such that the transcriptional interference between the stacked transgenes is minimal (Fig. 2). More recently, we reported on the feasibility of targeted molecular trait stacking in cotton (D'Halluin *et al.*, 2013). We showed precise integration of herbicide tolerance genes (*hppd*, *epsps*) in close vicinity of an already existing transgenic insect control locus (*cry2Ae*, *bar*) through the combination of targeted DNA cleavage by a custom-designed meganuclease and HR-mediated repair (Fig. 3). Hereto, a repair DNA was designed that carries the herbicide tolerance genes (*hppd*, *epsps*) flanked with cotton genomic sequences homologous to the DNA cleavage site. We further demonstrated that all genes in the resulting targeted molecular stack (*hppd*, *epsps*, *cry2Ae*, *bar*) were inherited as a single genetic locus, in a normal Mendelian manner. We observed that a large fraction of these precise stacked events did not contain additional random integrations of *hppd* or *epsps* or the meganuclease somewhere else in the genome. This is remarkable as our targeted molecular stacked events were

produced through particle bombardment-mediated DNA delivery of both the meganuclease and the repair DNA. Usually DNA delivery by biolistics is characterized by higher copy number integration (Hansen and Chilton, 1996; Travella *et al.*, 2005). We hypothesize that many of the repair proteins involved in DSB repair may be recruited to the nuclease-induced targeted DSB site thereby reducing the frequency of random integration.

Site-specific integration for trait stacking could also be mediated by site-specific recombinases such as *Cre* or *FLP* (Ow, 2011). However, site-specific recombination in the plant genome has only been possible at a pre-engineered recombination site and did not allow precise engineering at will at any desired position in the plant genome. Successful targeting of transgenes to pre-engineered recombination sites was reported for crop plants such as rice (Srivastava and Ow, 2001; Srivastava *et al.*, 2004; Chawla *et al.*, 2006) and soybean (Li *et al.*, 2009). Recently, considerable progress has been made in the engineering of zinc-finger recombinases and TALE recombinases. These enzymes consist of an activated catalytic domain derived from the resolvase/invertase

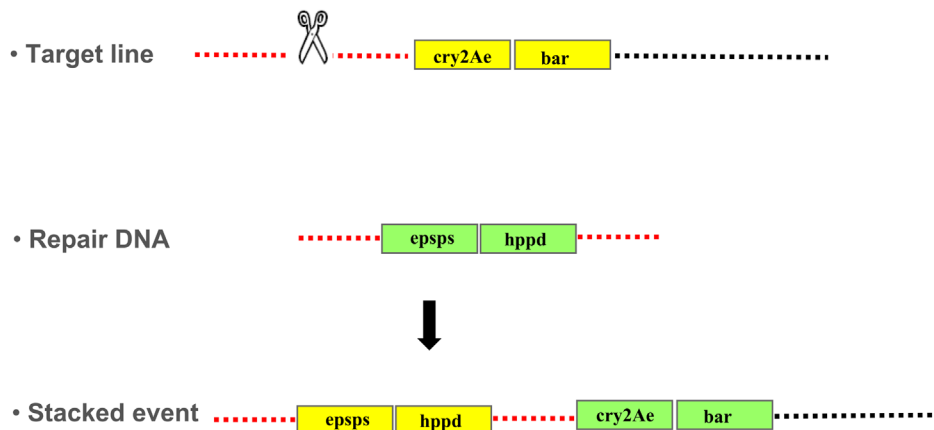


Fig. 3. Targeted molecular trait stacking in cotton through targeted double strand break induction. Precise integration of herbicide tolerance genes (*hppd*, *epsps*) (green boxes) in close vicinity of an already existing transgenic insect control locus (*cry2Ae*, *bar*) (yellow boxes) through targeted DNA cleavage by a sequence-specific nuclease (scissor) and HR-mediated repair. The red dotted lines show the regions of homology between the repair DNA and the target line. *hppd*, 4-hydroxyphenylpyruvate dioxygenase gene; *epsps*, 5-enol-pyruvylshikimate-3-phosphate synthase; *bar*, phosphinothricin acetyltransferase; *cry2Ae*, insecticidal crystal protein of *Bacillus thuringiensis*.

family of serine recombinases and a custom-designed zinc-finger- or TALE- binding domain. These designer recombinases were able to recombine DNA in mammalian cells (Mercer *et al.*, 2012; Gaj *et al.*, 2013). The feasibility of generating customized recombinases will also broaden the application range to endogenous sequences in plants.

Minichromosome technology has also been considered as a technology that may offer opportunities for trait stacking in crop plants. Artificial minichromosomes in maize have been produced by telomere-mediated truncation (Yu *et al.*, 2007) or by *in vitro* assembly from cloned centromeric like sequences (Carlson *et al.*, 2007). The latter authors reported that these *in vitro* assembled minichromosomes in maize transformants were maintained as an autonomous chromosome next to the host chromosomes and that the genes carried by the minichromosome were expressed and transmitted through mitosis and meiosis. Performance of these minichromosome transformants under field conditions has not yet been reported. Minichromosome transformation could have advantages over random integration into native chromosomes by circumventing the risk of random gene insertion into an undesirable chromosomal region which may result in linkage drag upon introgression of the transgene locus in other varieties.

Directed genome engineering for removal of sequences

Removal of sequences can be achieved by DSB-induced HR or NHEJ-mediated repair (Siebert and Puchta, 2002) or by site-specific intramolecular recombination (Odell *et al.*, 1990). DSB-mediated recombination allows removal of both transgene and endogene sequences, whereas site-specific recombination has been limited to transgene sequences preconfigured with recombination sites (Nanto *et al.*, 2009). An important application of both technologies for crop improvement is the removal of selectable marker genes only needed for transformation (Petolino *et al.*, 2010, Zhang *et al.*, 2003). The Cre/*loxP* recombination system has also been used to resolve complex integration patterns in transgenic wheat and maize plants after particle bombardment by removal of surplus copies (Srivastava and Ow, 2001).

The DSB-mediated approach has the ad-

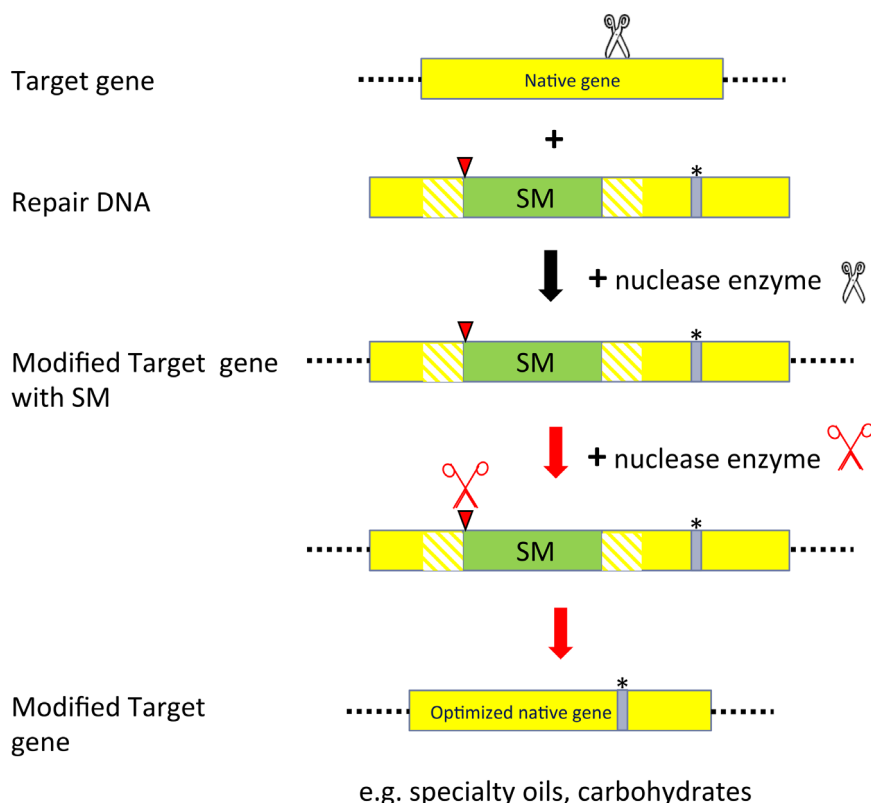
Fig. 4. Improving non-selectable native traits by temporal use of a selectable marker gene. A double strand break (DSB) is induced by a sequence-specific nuclease (black scissor) in the native gene (yellow box). The break is repaired by homologous recombination (HR) with a Repair DNA containing the desired sequence modification (*) to be incorporated in the Target gene and a selectable marker gene (SM) (green box) flanked by direct repeat sequences through duplication of part of the target native gene sequence (hatched yellow box). Between the direct repeat sequences, a recognition site (red triangle) for a sequence-specific nuclease (red scissor) is included. Induction of a DSB by the sequence-specific nuclease (red scissor) between the direct repeat sequences (yellow hatched box) may result in the removal of the selectable marker gene by intrachromosomal homologous recombination, leading to an optimized native gene containing only the desired modification (= Modified Target gene).

vantage over the recombinase-mediated approach to be also applicable for the selective removal of endogenous gene clusters or unwanted loci and also for the abolishment of gene function by site-directed mutagenesis as a result of small deletions by imperfect repair of a DSB.

Marker removal by DSB-induced intrachromosomal HR (IHR) may also become a very useful tool for temporary selection of otherwise non-selectable genome editing events. As a protoplast-based regeneration system is not available for most crop plants, genome editing of non-selectable genes will only be possible by using multicellular tissue culture systems. This will require at least temporarily the use of a selectable marker gene to enrich for tissues where genome editing may have happened. Once genome editing events have been identified, the selectable marker gene can be removed. The selectable marker gene could be removed without leaving any footprint to allow proper expression of the “edited” gene(s). This is possible by flanking the selectable marker gene by direct repeat sequences developed through duplication of part of the target DNA sequence. Upon the induction of a DSB between the repeats, the DSB will be repaired by IHR between the repeat sequences, resulting in the removal of the selectable marker gene without leaving any footprint (Fig. 4). We showed that by such an approach the selectable marker gene could be efficiently removed. By placing the endonuclease under control of the NTM19 microspore-specific promoter (Oldenhof *et al.*, 1996) we directly obtained progeny plants with precise removal of the marker gene and without leaving any footprint (WO06/105946).

Directed genome engineering for precise genome editing

DSB-induced HR-mediated repair is considered a useful tool



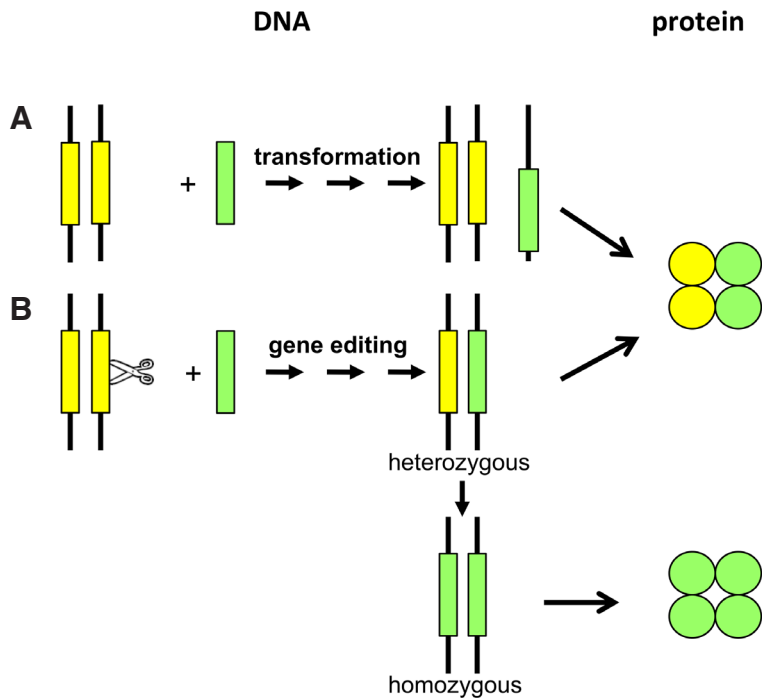


Fig. 5. Transformation versus genome editing for trait optimization. (A) Trait optimization through the introduction of an improved allele (green rectangle) by transformation. The yellow rectangle depicts the native allele. In transformants both wild type protein (yellow circles) and mutant protein (green circles) is present. (B) Trait optimization through genome editing by the induction of a targeted DSB (scissor) in the native allele and repair by homologous recombination. Homozygous genome editing events, contain only the mutant protein.

for the introduction of precise, subtle changes in genes. ZFNs and TALENs have been used for specific mutation induction in the tobacco acetolactate synthase genes (*ALS*) (*SurA* and *SurB*) conferring tolerance to the *ALS*-inhibiting herbicides (Townsend *et al.*, 2009; Zhang *et al.*, 2013). In these cases, the DSB was repaired using a repair DNA template that contains sequences homologous to the *ALS* target sequence with the desired mutation(s). Endo *et al.*, (2007) successfully introduced two mutations into the rice *ALS* gene via HR-mediated gene targeting but not triggered by a nuclease-mediated targeted DSB induction. These authors ascribe their success of gene targeting in absence of targeted DSB induction, mainly to their very efficient tissue culture and transformation system in rice and they further speculate that the condition of the chromatin structure of rice callus cells might enhance HR.

Rice plants with the two desired mutations introduced in the endogenous *ALS* gene showed hyper herbicide tolerance and were superior to “conventional” rice transformants that carried the same mutated *ALS* gene. Although the transformants had high expression of the mutant *ALS* gene, their tolerance was lower than that of the plants carrying the edited *ALS* native genes. This could be explained by the fact that in the transformants both wild type and mutant *ALS* protein is present while the homozygous *ALS* edited plants only contain the mutant herbicide tolerance providing *ALS* protein. (Fig. 5). This example shows that for the generation of plants tolerant to the *ALS*-inhibiting herbicides, precise genome editing may be preferred above transformation.

Another technology being explored for the introduction of subtle,

site-directed changes in chromosomal DNA is based on the use of oligonucleotides. This includes single stranded and double stranded oligonucleotides with different chemistry, and DNA-RNA hybrid molecules. Although some success has been obtained by oligonucleotide-driven mutagenesis in crop plants such as maize (Zhu *et al.*, 1999), rice (Koch-Evenko and Willmitzer, 2003; Okuzaki and Toriyama, 2004), and canola (BASF, 2009), this approach has not yet become routine and modifications were not always as intended, both with respect to position and to the base change (Beetham *et al.*, 1999). At least a part of the observed sequence modifications may also have been the result of spontaneous mutation (Ruiter *et al.*, 2003). The utility of oligonucleotides is restricted to the introduction of small modifications of a single or a few base pairs and have most often been applied for the creation of mutations in selectable traits such as the *ALS* genes to confer herbicide tolerance.

Directed genome engineering for structural non-precise changes

Repair of DSBs by NHEJ can introduce mutations at target sites by the creation of small deletions or insertions, resulting most typically in loss of gene function. This approach is technically relatively simple as it requires only the introduction of a targeted DSB and no repair DNA template is required. This makes it a powerful tool for gene function discovery. Targeted mutagenesis by targeted introduction of DSBs by designer nucleases and repair by NHEJ has already been described for several plant species, as in tobacco (Maeder *et al.*, 2008; Cermak *et al.*, 2011; Mahfouz *et al.*, 2011; Zhang *et al.*, 2012), and crop plants as soybean (Curtin *et al.*, 2011), maize (Gao *et al.*, 2010) and rice (Li *et al.*, 2012; Shan *et al.*, 2013). Recently it was shown that disease-resistant rice could be generated by site-directed mutagenesis of the rice bacterial blight susceptibility gene *Os11N3* through TALEN-mediated disruption of the effector-binding element for the TAL effector *AvrXa7* of *Xanthomonas oryzae* pv. *Oryzae* (Li *et al.*, 2012). This is the first example of the development of a trait in a crop plant through just the induction of a targeted DSB and repair in a random manner by NHEJ.

Conclusion

Directed genome engineering enables precise genome modification. It will become a powerful tool for developing new traits for crops, and for understanding and optimizing gene function. More and more crop genomes are being, or will soon be fully sequenced and characterized. For some crops, even domestic and wild varieties have been sequenced to find specific genetic variation between wild type and cultivated strains. Together with the continuously increasing information from systems biology, key genes or networks of genes that determine certain high value agronomic features will be identified. When the desired biodiversity for trait development is naturally present within the species, (molecular) breeding will be the desired tool towards crop improvement. If trait development is dependent on the addition of genes from other species, standard transformation will be required. In some cases though, the ability to precisely modify the genome will be required for effective trait

development, e.g. for introducing amino acid substitutions in a native gene to confer herbicide tolerance or increased tolerance to biotic or abiotic stresses or to optimize metabolic pathways to produce a variety of specialty products, such as oils, carbohydrates and plant-made pharmaceuticals.

From this brief review, it is clear that DSB-induced directed genome engineering is a very promising and versatile approach towards precise engineering of plant genomes. It can be applied for both small and large modifications including insertion, deletion and replacement of sequences. Although site-specific recombinases such as *Cre* or *Flp* have been demonstrated to mediate recombination in plants for integration, replacement and deletion of sequences, it has still been restricted to transgene sequences preconfigured with recombination sites. However, recent progress in the development of designer recombinases will broaden the application range to endogenous sequence. Time will tell whether oligonucleotide-mediated genome engineering which is limited to small modifications consisting of single or a few base pair changes, and the minichromosome technology, which is limited to transgene stacking will become broadly applicable genome engineering technology.

To make directed genome engineering broadly applicable for crop species, efficient DNA delivery methods and efficient cell and tissue culture procedures allowing the efficient manipulation of large numbers of cells have to be available. For many crop species, these have still to be further developed or improved.

As more complex traits are being aimed for optimization, directed genome engineering technology will be needed to cleave at specific locations in the genome to disable or edit specific genes. The progress in the field of the site-specific nucleases has opened the door for genome editing of crop plants.

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