


## SI EDITOR'S CHOICE

# From classical mutagenesis to nuclease-based breeding – directing natural DNA repair for a natural end-product

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## SUMMARY

Production of mutants of crop plants by the use of chemical or physical genotoxins has a long tradition. These factors induce the natural DNA repair machinery to repair damage in an error-prone way. In the case of radiation, multiple double-strand breaks (DSBs) are induced randomly in the genome, leading in very rare cases to a desirable phenotype. In recent years the use of synthetic, site-directed nucleases (SDNs) – also referred to as sequence-specific nucleases – like the CRISPR/Cas system has enabled scientists to use exactly the same naturally occurring DNA repair mechanisms for the controlled induction of genomic changes at pre-defined sites in plant genomes. As these changes are not necessarily associated with the permanent integration of foreign DNA, the obtained organisms *per se* cannot be regarded as genetically modified as there is no way to distinguish them from natural variants. This applies to changes induced by DSBs as well as single-strand breaks, and involves repair by non-homologous end-joining and homologous recombination. The recent development of SDN-based 'DNA-free' approaches makes mutagenesis strategies in classical breeding indistinguishable from SDN-derived targeted genome modifications, even in regard to current regulatory rules. With the advent of new SDN technologies, much faster and more precise genome editing becomes available at reasonable cost, and potentially without requiring time-consuming deregulation of newly created phenotypes. This review will focus on classical mutagenesis breeding and the application of newly developed SDNs in order to emphasize similarities in the context of the regulatory situation for genetically modified crop plants.

**Keywords:** genome engineering, synthetic DNA nucleases, gene editing, non-homologous end-joining.

## INTRODUCTION

Since the beginning of life, the genetic code has been subject to natural changes, known as mutations. These mutations occur due to error-prone repair mechanisms and physical or chemical factors that interact with the genetic material. Such modifications of the molecules encoding the properties of living beings such as humans, plants, algae, fungi, bacteria and archaeobacteria, affect DNA as well as DNA- and RNA-encoded viruses in their host cells. Various naturally occurring factors such as ultraviolet (UV) irradiation and reactive oxygen species (ROS) can make a significant contribution, and still continue to contribute to the modification of the genetic information. Due to such natural modifications, small-scale changes occur in the

genetic repertoire of species over time. Depending on their advantage or disadvantage for the survival of the respective organism, under given environmental conditions, certain mutations may prove advantageous or disadvantageous. Under selective pressure, mutations which prove to be advantageous for survival will become enriched in a species. In this way, selection pressure shapes the evolution of life forms and viruses, adapting them at all times to better fit to the environment. Furthermore, the genes encoding the proteins involved in DNA repair pathways are also under selective pressure to adapt the cell or organism to genome-damaging environmental factors. Due to the plasticity of the plant genome, namely the late

determination of the germline, mutations are first challenged in somatic tissues and eventually find their way into the germline.

While the above-mentioned natural mutations occur unintentionally and randomly, humans recognized early the possibility of selective breeding, which is the combination of advantageous traits of the same species, in order to obtain desired properties. When humans changed from being nomadic hunters to having a sessile life-style, a transition dated to the start of the Neolithic around 11 000 BC, people started growing plants using simple agricultural methods (Gepts, 2001). Noticing properties like higher yield or enhanced endurance under difficult environmental conditions, those early farmers probably unconsciously started selecting individual plants with superior traits or performance. In this way, people had already begun very early on to influence plant genome evolution in a way that was advantageous for human nutrition. Without any knowledge about genetics, this was a very time-consuming and labor-intensive process. Nevertheless, even these ancient efforts led to improved yield, pest resistance, drought and heat tolerance, as well as increased tolerance to water stress. Due to this human-induced selective evolutionary pressure, many of the original natural species became lost and only a small number of crop species are now important for human nutrition. Furthermore, due to selective breeding, large parts of crop genomes are essentially fixed, and thus reshuffling of traits for further breeding of new variants is limited. Interestingly, the advantage of breeding to meet the nutritional demands of the population is said to have been a major driver for the Industrial Revolution (Gepts, 2002). In modern times, with a continuously growing world population and the fact that land suitable for growing crop plants is finite, it is obvious that further improvements are required to meet future human needs.

Following a short section about the discovery of mutagenic factors and their relevance for plant breeding and basic science, this review focuses on specific DNA repair pathways which are of major importance for understanding how state-of-the-art genome editing (GE) tools can be applied. Further, we will provide an overview about the current GE technologies and summarize how they can be classified in order to facilitate regulatory concerns. Finally, we will provide an outlook about the perspectives of GE compared with classical mutational breeding and conclude with an assessment of how legislative improvements may lower the hurdles for commercialization of agronomically important traits.

## CLASSICAL MUTAGENESIS

Hugo de Vries (1901, 1903, 1905) was the first to suggest that X-rays and gamma rays (newly discovered by Konrad von Roentgen in 1895 and Henry Becquerel in 1896 and

Pierre and Marie Curie in 1897–8, respectively) might be of great scientific importance for artificially inducing mutations:

A knowledge of the laws of mutation must sooner or later lead to the possibility of inducing mutations at will and so of originating perfectly new characters in animals and plants. And just as the process of selection has enabled us to produce improved races, greater in value and in beauty, so a control of the mutative process will, it is hoped, place in our hands the power of originating permanently improved species of animals and plants. de Vries (1909)

In the early 20th century, the first discoveries were made by Muller (1927) and Stadler (1928), who described the induction of mutations by X-rays in *Drosophila*, maize, barley and wheat. With those initial findings, for which Muller was awarded the Nobel Prize for Physiology or Medicine in 1946, the basis was laid for many years of successful mutation breeding. Since then, radiation-induced mutants have been created and studied extensively for the analysis of gene function, the creation of novel traits of agricultural importance and the study of DNA repair mechanisms (Shu *et al.*, 2011). There are more than 3200 mutant varieties officially released for commercial use in more than 210 plant species, from more than 70 countries, as referenced in the Mutant Varieties Database (<https://mvd.iaea.org>).

In the early 1960s researchers discovered the potential to increase the rate of such undirected genome modifications using gamma rays in order to accelerate mutation breeding processes. One well-known example is the Gamma Field of the Japanese Institute of Radiation Breeding (Nakagawa, 2009). Both gamma rays and X-rays are highly energetic and can directly or indirectly, via the generation of reactive oxygen species (ROS), cause damage to nucleic acids. Plants can be exposed to different doses of radiation by varying the distance to the radiation source or varying the exposure time; therefore the number of mutations per cell can differ significantly. Due to the random nature of those mutations, screening and selection processes to identify mutants with superior traits are regarded as the main challenge in radiation breeding.

The first experiments on chemical mutagenesis were performed by Thom and Steinberger in 1939 using nitrous acid in *Aspergillus*. In 1941, Charlotte Auerbach was the first to describe that mustard gas had a mutagenic effect on *Drosophila* (Auerbach, 1941; Beale, 1993). Auerbach and Robson (1946) found that the mutagenic effects of mustard gas are comparable to the effects of X-rays, resulting in gene mutations, insertions, deletions or translocations. While those experiments were conducted with *Drosophila*, Oehlker (1943) and Gustafsson and Mackey (1948) confirmed that mustard gas is also highly mutagenic in barley. Rapoport (1946, 1948) established that

alkylating agents are the most important group of chemical mutagens. Following Watson and Crick's identification of DNA as the basis of the genetic code in 1953, precise planning of experiments was permitted enabling the underlying mechanisms of mutations to be understood. While Stadler (1928) was still skeptical about the potential of mutation breeding, the first economically relevant mutant crops were described in the 1930s and 1940s in wheat (Sapehin, 1930), *Antirrhinum* (Stubbe, 1934), tobacco 'Chlorina mutant' (Tollenaar, 1934) and barley (with X-ray induced mildew resistance; Freisleben and Lein, 1942). A Swedish mutation program led by A. Gustafsson gave additional insights into plant mutation breeding (Lundqvist, 2009). Subsequently, several countries began efforts to improve crop species with new mutagenesis programs (Kharkwal *et al.*, 2004).

While the initial focus of plant mutagenesis programs was to improve traits like yield, disease or pest resistances in various crop species, newer approaches shifted towards the improvement of quality and nutritional value, as well as tolerance to abiotic stress factors.

Next to physical mutagenesis factors and the initial experiments with mustard gas, as discussed above, a large number of chemical mutagenic compounds are now known; however, only a few have been applied in plant mutagenesis. Over 80% of the mutagens applied in plants are alkylating agents, like ethylmethanesulfonate (EMS), methylnitrosourea (MNU) and ethylnitrosourea (ENU). EMS is quite reactive as an alkylating agent; its ethyl group reacts with the guanine bases in DNA, forming the abnormal base  $O^6$ -ethylguanine, for example. During replication, DNA polymerases frequently place thymine, instead of cytosine, opposite  $O^6$ -ethylguanine. Following subsequent rounds of replication, the original G:C base pair can become an A:T pair (transition mutation), catalyzed by  $O^6$ -alkylguanine alkyltransferase. In this way, EMS has been used to randomly induce point mutations in many species for forward genetics and for the generation of agronomically relevant traits (Dhaliwal *et al.*, 2015).

Treatment with UV light, X-ray irradiation or EMS causes a significant increase in genome damage and the resulting modifications. In this way, the mutation rate was dramatically increased above natural levels. Nevertheless, all such human-induced changes were absolutely uncontrolled and modified the respective genomes at countless positions in an undirected way, requiring sophisticated screening and selection procedures. However, for breeding purposes, the main interest and major drive is to improve the properties of the respective species independent of any other accidentally occurring changes in the genome, a challenge that can now be addressed for the first time in human history by the use of SDN-mediated genome engineering.

As discussed above, both natural agents, like UV radiation, and chemically reactive species, like ROS, human-

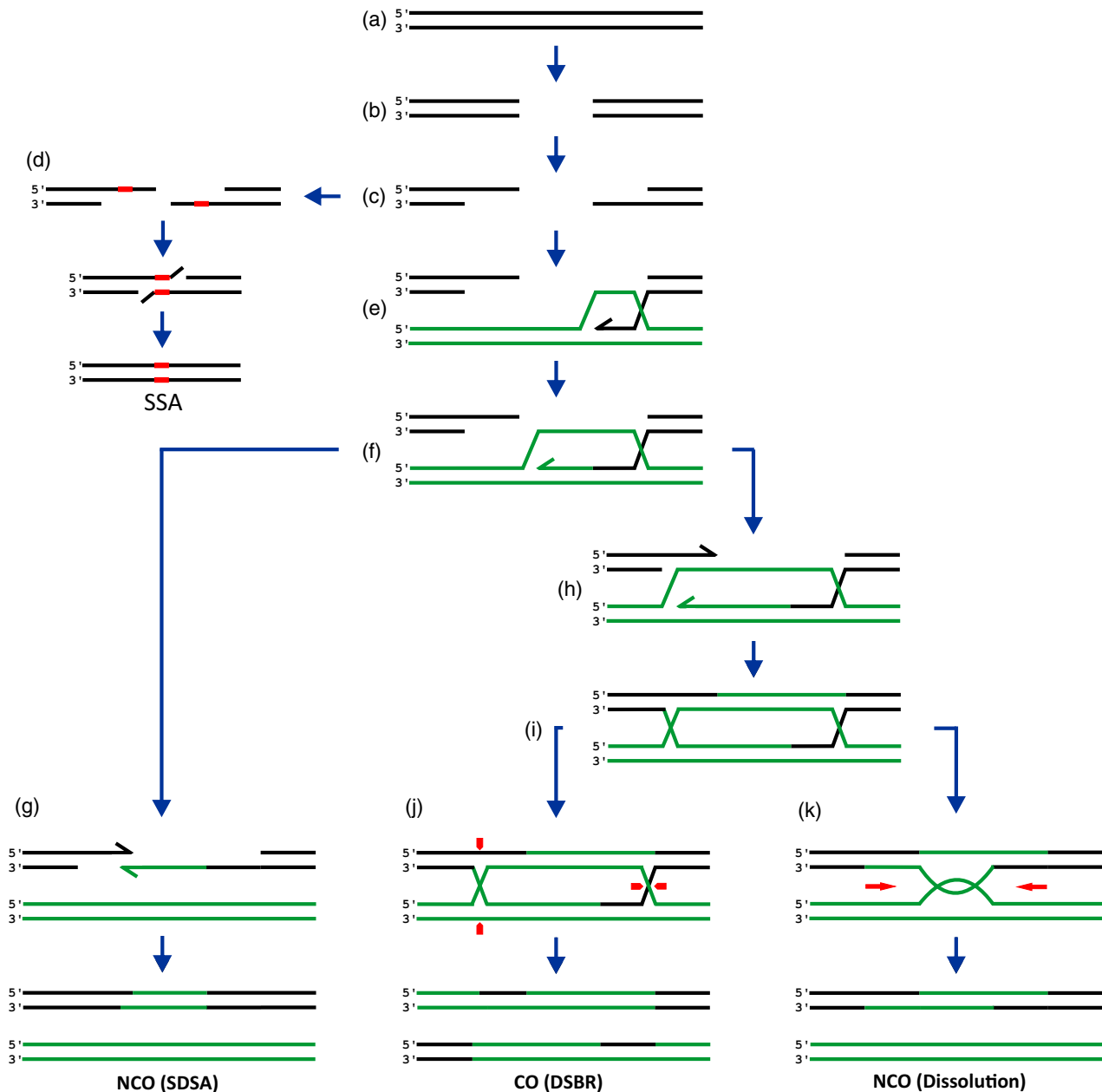
created X-rays and EMS treatments, cause physical or chemical damage to nucleic acids, the carriers of genetic information. As DNA-damaging agents existed in the environment when living beings and viruses emerged on Earth, there has always been selection pressure to repair such damage in order to ensure the survival of cells or organisms. Therefore, repair pathways evolved as defense mechanisms to protect the integrity of the genetic information, whilst still allowing for a certain degree of mutation. The latter is a requirement to keep the genome sufficiently dynamic that new – potentially advantageous phenotypes – can arise in order to allow an adaptive evolution on a cellular and organism level. These repair mechanisms are completely natural and contribute to the successful survival of all species.

### DNA REPAIR PATHWAYS UTILIZED FOR GENOME EDITING

As discussed above, classical physico-chemical mutagenesis breeding relies on *randomly* induced mutations, deletions or genome rearrangements. With the advent of new molecular biology tools (see below), such genome modifications can be *precisely* induced in order to generate new valuable traits. The major pathway for GE is DSB repair (DSBR). It has long been established that the induction of a DSB triggers highly specialized repair pathways. In general, DSBs can be repaired via two major pathways – homologous recombination (HR) or non-homologous end-joining (NHEJ). While NHEJ is the predominant DSB repair pathway in somatic plant cells, HR is highly efficient in meiotic tissues, supporting the genetic exchange between the parental homologous chromosomes. Independent of the pathway, the initial step is the occurrence of a DSB. Therefore, it is essential to understand the basis of DSB-induced genome modifications *in planta* (Puchta, 2005).

Three mechanisms of HR have been characterized: single-strand annealing (SSA), synthesis-dependent strand annealing (SDSA) and the so-called classical DSBR model (Figure 1). DSBR is a mechanism that describes the repair of DSBs during meiotic recombination (Osman *et al.*, 2011).

Upon DSB induction (Figure 1b), single-stranded DNA overhangs are produced via exonuclease-catalyzed resection (Figure 1c). If both ends possess significant homologies, direct annealing via the SSA mechanism may occur (Figure 1d). Consequently, sequence information between the homologies will be lost. SSA is an efficient mechanism which has been shown in tobacco by site-directed DSB induction in a transgenic substrate (Siebert and Puchta, 2002). SSA can also occur between unlinked homologies (Puchta and Hohn, 1991; Tinland *et al.*, 1994; Pacher *et al.*, 2007). In the case of DSBR and SDSA, 3' end invasion of a single strand into a homologous donor template occurs, resulting in a D-loop structure, and the newly paired strand is used for repair synthesis (Figure 1e,f). For SDSA, the



**Figure 1.** Mechanisms of homologous recombination (HR).

The HR-mediated repair of double-strand breaks (DSBs) can occur via three different pathways. Subsequent to the occurrence of a DSB (b) in a double-stranded DNA sequence (a), exonuclease-catalyzed end resection yields single-stranded 3' overhangs (c). If sequence homologies between the two resected ends are present (highlighted in red), annealing can happen (d) via the single-strand annealing (SSA) mechanism. Subsequently, 3' overhangs are resected and single-strand gaps filled out through DNA synthesis. Consequently, sequence information between those homologies is lost. Therefore, the SSA pathway is also described as a non-conservative HR DSB repair mechanism. In the absence of such homologies, the conservative HR pathways can mediate the DSB repair (DSBR). A D-loop structure is formed by invasion of a resected 3' single strand (e) using a homologous strand as a matrix (f) for copying sequence information. In the case of the synthesis-dependent strand annealing (SDSA) mechanism (g) only sequence information is copied from the homologous donor matrix to the invaded strand. Following D-loop displacement, end adjustment, repair synthesis and ligation to the other broken end, SDSA leads to a non-crossover (NCO) event. In the case of HR-mediated repair via the DSBR model (h), next to the DNA synthesis in the D-loop, also the other free 3' end undergoes repair synthesis using the displaced strand of the donor molecule as template, forming a double Holliday junction (dHJ), depicted in (i). Depending on the orientation of the dHJ processing enzymes, resolution or dissolution of the dHJ lead to (j) crossover (CO) or (k) NCO events.

genetic information from the donor matrix is copied to the damaged strand (Figure 1g), while in the DSBR pathway DNA synthesis also occurs at the other broken end,

resulting in information being copied from both ends of the matrix (Figure 1h,i). During SDSA, the extended strand hybridizes with a single strand from the other end of the

resected break, which consequently results in gene conversion, whereas during DSBR a double Holliday junction (dHJ) is formed. Depending on the processing of the dHJ structure, resolution or dissolution, the outcome of genetic exchange varies (Figure 1j,k). Resolution leads to crossover events and can thus recombine larger chromosome parts. The latter mechanism is of significant importance for meiotic recombination of parental genomes (Osman *et al.*, 2011). HR in the SDSA mechanism can occur with homologies at only one end of the DSB [one-side invasion (OSI) model] (Belmaaza and Chartrand, 1994; Puchta, 1998). OSI is also able to explain combinations of HR and NHEJ reactions at DSB sites, which are frequently observed. DSBR is thought to be the major DSBR pathway during meiosis, and its limitation during somatic recombination prevents crossover frequencies from becoming too high, which would result in di- and acentric chromosomes. This is due to multiple ectopic homologies in the plant genome being used as templates for HR, which can be (i) intrachromosomal homologies, (ii) the sister chromatid, (iii) allelic sequences in diploid cells, or (iv) homologous sequences in ectopic positions (Puchta and Fauser, 2015). The SDSA model supports the explanation of observed gene targeting (GT) experiments in somatic plant cells. Many combinations of HR (OSI) and NHEJ have been described to date (Puchta and Fauser, 2013). Negative selectable markers have been used to prevent OSI events in favor of both side HR mediated GT (Zhang *et al.*, 2015).

While the HR-mediated DSBR discussed above describes interactions between naturally occurring genetic sequences within or between stably inserted transgenic sequences, those mechanisms can also be exploited by making use of extrachromosomal templates provided, for example, via an incoming T-DNA from *Agrobacterium tumefaciens*, transfected plasmids, biolistic delivery of DNA sequences or delivery by viral replicons. Twenty years ago, it was already established that an incoming T-DNA with homologies to a genomic locus is, by several orders of magnitude, a better HR template than an ectopic chromosomal site carrying the same homologies (Puchta *et al.*, 1996; Puchta, 1998). It is thought that the observed higher recombination efficiency is correlated with less steric hindrance than a chromatin-associated ectopic sequence. Furthermore, it could also be demonstrated that a linear DNA fragment excised *in vivo* with a site-specific nuclease is an efficient template for *in planta* gene targeting (GT), in both transgenic (Fauser *et al.*, 2012) and natural loci (Fauser *et al.*, 2014). HR-mediated *in planta* GT allows for small-scale modifications by using targeting vectors with significant flanking sequences (Fauser *et al.*, 2014), or alternatively introducing new genes between the homologies (Fauser *et al.*, 2012). The use of DNA-based Geminiviruses may increase the carrying potential for sequences to be introduced for GT. As Geminiviruses form

replicons in the plant nucleus, high copies of the introduced DNA fragment can be accumulated via the rolling circle process, serving as a repair template at a site-directed DSB. In this way, many donor molecules can be generated in an individual cell and thus increase the probability of HR-mediated DSBR, especially if a SDN is delivered to the same molecule (Baltes *et al.*, 2014; Cermak *et al.*, 2015).

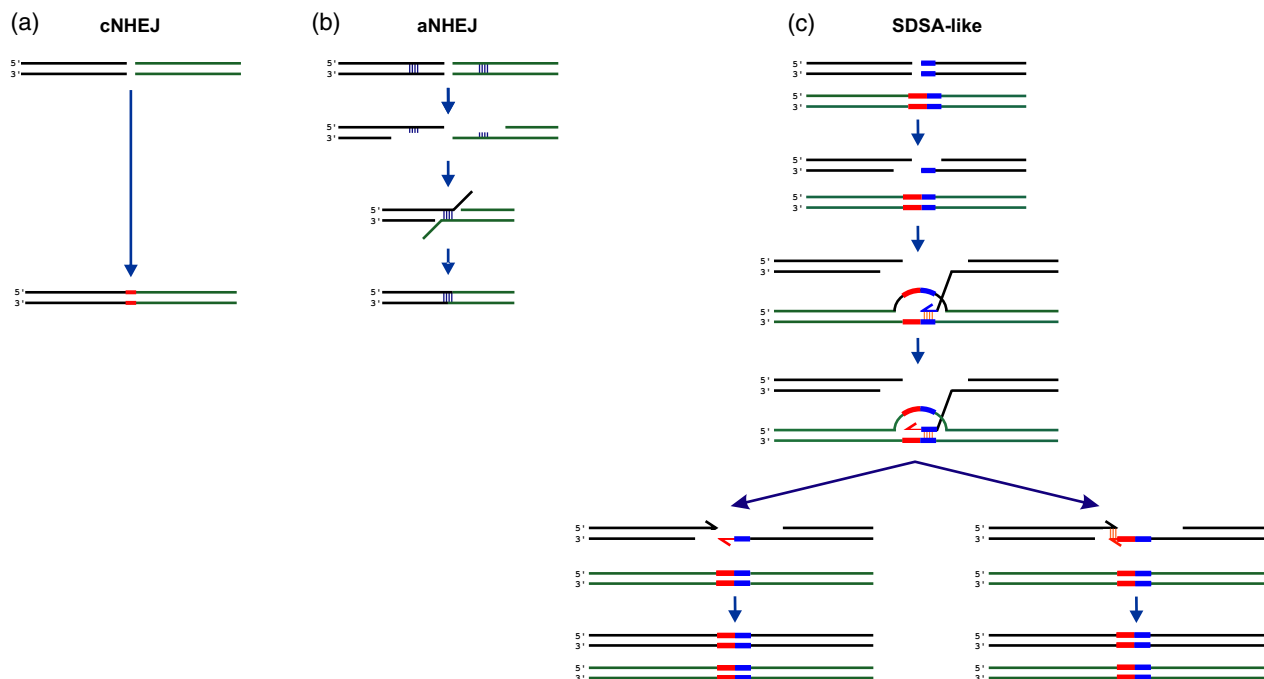
Next to HR-mediated DSBR, the main mechanism of DSBR in somatic plant cells, NHEJ, can also be exploited to integrate DNA at predetermined sites when using SDNs, or to achieve targeted mutagenesis (Salomon and Puchta, 1998; Li *et al.*, 2016). DSBR via NHEJ typically does not require any homologous sequences for the repair reaction (Figure 2a,b). Ends can be ligated or small insertion or deletions can occur. The NHEJ mechanism ensures an efficient DSBR without significant sequence loss; however, in the case of multiple DSBs at the same time in one genome, rearrangements can take place. So far, two NHEJ pathways have been described in plants. The classical NHEJ (cNHEJ) pathway involves minimal end processing before ligation (Figure 2a). As the end resection before ligation can result in a few nucleotide deletions or insertions, the repair pathway can be mutagenic when occurring in an open reading frame (ORF).

The alternative NHEJ (aNHEJ) pathway (Mladenov and Iliakis, 2011) mechanistically resembles, to a certain extent, the SSA pathway discussed above (Figure 2b). In this case, small microhomologies are annealed after resection and trimming of the 3' ends. Consequently, aNHEJ leads to the deletion of genetic information and has a higher mutagenic potential than cNHEJ. Moreover, a micro-homology-based SDSA-like mechanism also explains the copying of insertions from elsewhere in the genome into the DSB (Figure 2c) (Salomon and Puchta, 1998).

The application of NHEJ for genome engineering is not only limited to the generation of targeted mutations or sequence insertions, as simultaneous induction of several DSBs can allow for the breaking of genetic linkage groups or reshuffling entire chromosome orientations (Le Cong *et al.*, 2013; Li *et al.*, 2013; Mali *et al.*, 2013; Wang *et al.*, 2013). With the advent of SDNs, it became possible to induce multiple simultaneous DSBs at different loci to excise genomic sequences (Siebert and Puchta, 2002; Petolino *et al.*, 2010), create inversions (Lee *et al.*, 2012), permit reciprocal chromosomal translocations (Pacher *et al.*, 2007) or exchange chromosome fragments (Weinthal *et al.*, 2013).

## NEW TOOLS FOR PRECISE NATURAL GENOME ENGINEERING

Natural mutations and mutational breeding act randomly on the plant genome, making the outcome unpredictable. However, in recent decades molecular biologists



**Figure 2.** Mechanisms of non-homologous end joining (NHEJ) and synthesis-dependent strand annealing (SDSA)-like capture of sequences. At least two different pathways for double-strand break (DSB) repair by NHEJ operate in plant cells. (a) The characteristics of the canonical NHEJ (cNHEJ) are minimal processing of the broken ends prior to ligation. Typically, only limited sequence information is lost or inserted and no microhomologies are involved. (b) In contrast, in the alternative NHEJ (aNHEJ) pathway, the processing of broken ends is more pronounced and microhomologies typically mediate the ligation of the free ends. Therefore, aNHEJ mechanistically resembles the HR-mediated single-strand annealing repair pathway and deletions are often observed following the repair. (c) SDSA-like insertions are observed after DSB induction when single-stranded overhangs are produced that invade via the 3' end into microhomologies (blue) elsewhere in the genome. Following strand displacement DSB repair can occur via cNHEJ or aNHEJ, copying additional sequence information (red) into the original locus.

discovered SSNs/SDNs [the latter term having been created by the European Food Safety Authority (EFSA) panel on genetically modified organisms (GMOs) in 2012 (Waigmann *et al.*, 2012)]; these have high specificity, and scientists have learned how to program them to target sequences of interest. Having those molecular tools at hand, site-directed GE became more and more feasible and off-site effects could be significantly reduced (for reviews see Voytas, 2013; Puchta and Fauser, 2014; Chandrasegaran and Carroll, 2016).

### Meganucleases, zinc finger nucleases and TALE nucleases

Meganucleases (MEs) were the first naturally existing tools to be exploited for targeted induction of DSBs in various organisms. Due to their long DNA target sequences, between 18 and 40 bp, MEs have high sequence specificity. A row of experiments for targeted mutagenesis and GT have been performed *in planta* with naturally occurring MEs such as I-SceI (Puchta *et al.*, 1993) and I-CreI (Rosen *et al.*, 2006) as well as with designed MEs (D'Halluin *et al.*, 2013). Their natural target sequences limit applications mainly to targeted DSBs in inserted transgenic sequences, and genetic manipulation of the recognition site is extremely labor-intensive with the outcome being hard to

predict (Steuer *et al.*, 2004; Arnould *et al.*, 2011). Furthermore, some MEs can have reduced sequence specificity allowing for off-target induced DSBs, which is highly unfavorable.

The introduction of zinc finger nucleases (ZFNs) represented a major improvement (Kim *et al.*, 1996; Smith, 2000). With the fusion of the DNA cleavage domain of the restriction enzyme *FokI* to the highly variable DNA-binding domain (DBD) of a class of zinc finger transcription factors, it became possible for the first time to artificially combine ZFN DBDs to determine the DNA cleavage site. As cloning of ZFNs is quite time-consuming and not all ZFN arrays work comparably well, and thus require extensive testing, ZFN technology has not turned out to be as successful as originally hoped.

Another new and important class of engineered nucleases are the transcription activator-like effectors (TALEs) from the plant pathogen *Xanthomonas* (Bonas *et al.*, 1989). The DBD of TALEs consists of numerous repeats varying by only two amino acids. Following the discovery that each of the repeating sequences binds to exactly one nucleotide of the DNA, engineered TALEs with *FokI* fusions were created (Boch *et al.*, 2009; Moscou and Bogdanove, 2009). GoldenGate-mediated cloning facilitated the

engineering of new sequence specific variants of TALE nucleases (TALENs) (Cermak *et al.*, 2011).

### CRISPR/Cas9

While TALENs played, and still play, an important role in plant GE, the discovery of a new class of programmable nucleases provides unprecedented flexibility, precision and speed to alter basically every possible target sequence in a simple way. Clustered regularly interspaced short palindromic repeats (CRISPR), which are naturally occurring as an adaptive bacterial immune system, were first described in the 1980s in *Escherichia coli* (Stern *et al.*, 1984; Ishino *et al.*, 1987). Meanwhile this system for defense against phages has also been discovered in various bacteria (e.g. *Streptococcus pyogenes*, *Staphylococcus aureus*) and archaeobacteria (Wiedenheft *et al.*, 2012). In 2012, the molecular mechanism of this system was deciphered (Jinek *et al.*, 2012): foreign DNA entering the bacterial cells is degraded by the Cas1 and Cas2 enzymes and inserted into the CRISPR locus. The Cas9 nuclease target specificity is governed by the CRISPR-RNA (crRNA), encoded by the CRISPR locus, and is complementary to the invading DNA with 20 nucleotides of homology. Additionally, the bacterial protospacer adjacent motif (PAM) is required for the recognition of the target site. A second short RNA (tracrRNA) binds to the crRNA and thus forms a stable complex with Cas9. Following binding of the target sequence, the two nuclease domains of Cas9, RuvC and HNH, cut the intruding DNA (Figure 3a). Artificially, the two naturally occurring RNAs (crRNA and tracrRNA) can be fused to form one molecule, called a single-guide RNA (sgRNA) (Jinek *et al.*, 2012). Given the fact of RNA–DNA sequence complementarity and the resulting specificity, the CRISPR/Cas system became the most useful DNA scissor (Doudna and Charpentier, 2014). The utility of CRISPR/Cas for targeted gene editing and GE has been proved in

many studies in plants (Wang *et al.*, 2014; Bortesi and Fischer, 2015; Schaeffer and Nakata, 2015; Hilscher *et al.*, 2016; Ma *et al.*, 2016; Puchta, 2016, 2017; Quetier, 2016; Samanta *et al.*, 2016; Schiml and Puchta, 2016; Steinert *et al.*, 2016). Furthermore, multiplex GE has been demonstrated in rice (Ma and Liu, 2016) and Arabidopsis (Xing *et al.*, 2014).

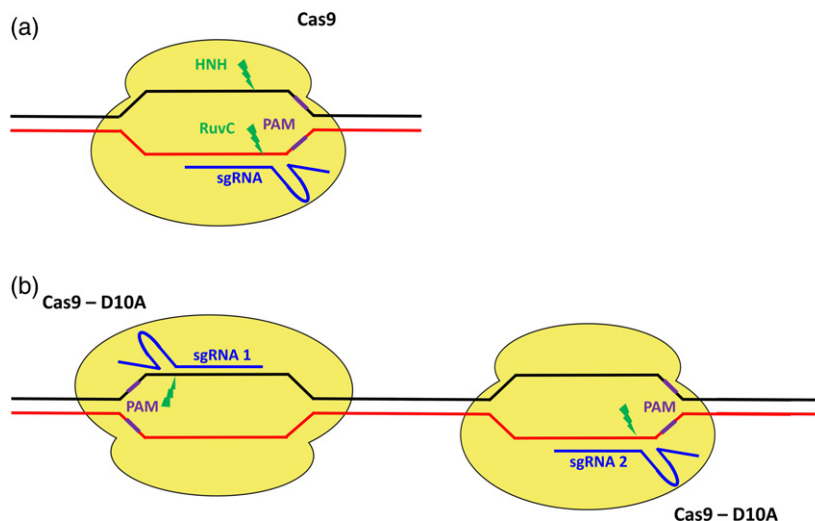
However, off-target effects can pose an obstacle for MEs, ZFNs and TALENs, with various studies *in planta* so far leading to contradictory findings, with no observed off-target activities being reported in some cases (Li *et al.*, 2013; Nekrasov *et al.*, 2013; Shan *et al.*, 2013; Upadhyay *et al.*, 2013; Feng *et al.*, 2014; Jia and Wang, 2014) while such activities were described in others (Endo *et al.*, 2015). In order to reduce such possible off-target activities if required, two recent technical developments can be applied. Artificial high-fidelity Cas9 enzymes have been tested which are more specific than the natural enzyme (Kleinstiver *et al.*, 2016). Furthermore, it is possible to inactivate one of the DNA cleavage sites of Cas9 with a point mutation creating a nickase, which is a SSB-inducing enzyme. Using one nickase with two sgRNAs, paired SSBs can be induced at defined distances thus increasing the sequence specificity (Ran *et al.*, 2013). This approach was successfully applied *in planta* (Schiml *et al.*, 2014, 2016; Mikami *et al.*, 2016) (Figure 3b).

Additionally, the availability of different Cas9 orthologues allows for even more complex GE approaches, as the species-specific PAM sequences together with the respective Cas9 orthologues can be applied simultaneously in the same genome without interference (Steinert *et al.*, 2015). It has also been demonstrated that Cas9 from *Staphylococcus aureus* has a higher binding specificity than the one from *Streptococcus pyogenes* (Kaya *et al.*, 2016). Additionally, the discovery of an alternative CRISPR-like system, Cpf1, in *Francisella novicida* (Kleinstiver *et al.*,

**Figure 3.** CRISPR/Cas9 – an efficient tool for precise genome engineering.

(a) Schematic representation of the Cas9 cleavage mechanism. The Cas9/single-guide RNA (sgRNA) complex recognizes and binds the complementary sequence next to the protospacer adjacent motif (PAM), which is highly specific for each Cas9 from different bacterial species. The RuvC domain and HNH motif of Cas9 cleave the two DNA strands 3 bp upstream of the PAM.

(b) Cas9 paired nickase approach. By using two sgRNAs, the D10A nickase variant can be guided to two opposite DNA strands at adjacent positions. The resulting double-strand break exhibits long single-stranded 5' overhangs.



2016), increases the possibilities of targeted genome modifications. While Cas9 creates blunt-end DSBs proximal to a G-rich PAM, Cpf1 generates sticky ends with 5' overhangs distal to a T-rich PAM, which might be of additional use to enhance ligation during GT approaches. A recent report showed that Cpf1 from *F. novicida* is an effective tool for targeted mutagenesis *in planta* (Endo *et al.*, 2016). Another group has most recently demonstrated the use of an alternative CRISPR–Cpf1 system (LbCpf1) for the targeted generation of mutant rice (Xu *et al.*, 2016). Interestingly, the PAM sequence of LbCpf1 (TTTN) is longer than the one of FnCpf1 (TTN). Thus, it is thought that the frequency of target sequences for FnCpf1 in plant genomes is higher than for LbCpf1.

### Oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis (ODM) does not require a SDN nor the formation of a DSB. With ODMs, small oligonucleotides are delivered into the host cell and bind via homology to the unwound helix target site, with small mismatches. As a result of DNA repair, the endogenous strand is corrected and base changes or small INDELS (insertions/deletions) can be incorporated into the genome. Upon completion of the repair process, the ODM is degraded through natural cellular processes. Therefore, the advantage of the ODM approach is that it is non-transgenic and exploits the existing DNA repair pathways (Dong *et al.*, 2006; Sauer *et al.*, 2016a). ODM-mediated mutagenesis has been successfully applied in tobacco (Beetham *et al.*, 1999; Ruitter *et al.*, 2003), *Zea mays* (Zhu *et al.*, 1999, 2000), Arabidopsis (Kochevenko and Willmitzer, 2003), rice (Okuzaki and Toriyama, 2004) and *Brassica napus* (Ruitter *et al.*, 2003), delivering the ODM via biolistic transformation or PEG-mediated protoplast transformation. However, mutation frequencies are low compared with SDNs and therefore both approaches have recently been combined to increase efficiency (Sauer *et al.*, 2016b).

### Base editing and beyond

Targeted nucleotide editing is another approach which has proved successful in yeast and mammalian cells (Komor *et al.*, 2016; Nishida *et al.*, 2016) as well as in rice (Lu and Zhu, 2016). Nuclease-inactivated Cas9 (dCas9) was fused to a cytidine deaminase which mediates the conversion of cytosine to uracil. In following replications, the C–G base pair is changed to a T–A base pair, resulting in a transition mutation. This method allows targeted introduction of point mutations even without DSB induction, in a tight 5-bp range close to the dCas9-binding site. Furthermore, dCas9 can be fused to transcriptional enhancers or repressors for regulation of gene expression, or to modify the epigenetic code via histone methylation/demethylation (for review see Puchta, 2016, 2017; Wang *et al.*, 2016).

## CLASSIFICATION OF GENE AND GENOME EDITING EVENTS

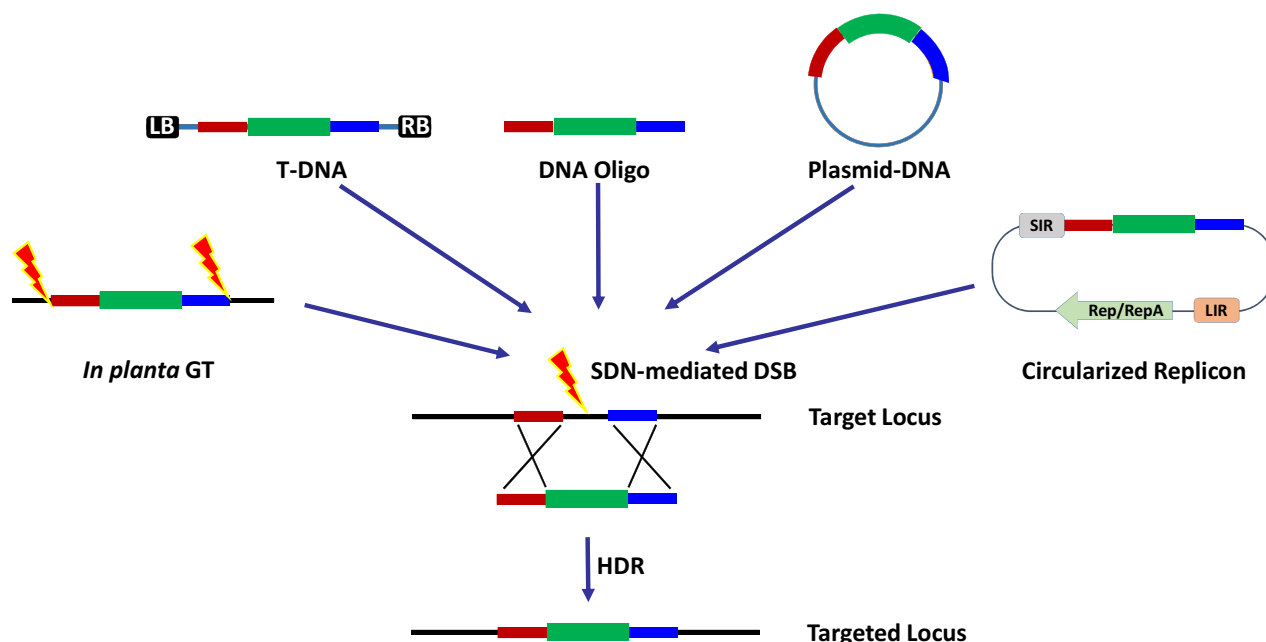
Based on the discussed the possibilities for GE it is evident that quite a range of different tools is currently available to modify plant genomes at targeted positions with high efficiency. However, from a commercial point of view, even these discussed high-precision tools are of limited relevance for revolutionizing plant breeding in order to achieve new agronomically valuable traits if highly time-consuming and costly deregulation processes delay market introductions and thus decrease the return of value. Therefore, it is of significant importance to consider the different approaches to GE and how they are currently perceived by regulating agencies in different countries.

Currently, three different categories for SDN-mediated genome modifications have been defined (Podevin *et al.*, 2013; Hilscher *et al.*, 2016) based on the European Union (EU) New Techniques Working Group (NTWG; European Commission *et al.*) classification of ZFN activity and regulatory purposes:

- (i) SDN1 covers the application of a SDN without an additional donor DNA or repair template. Thus the reaction outcome clearly depends on the DSB repair pathway of the plant genome. As the predominant DSB repair pathway is NHEJ, small insertions or deletions can occur (SDN1a). In the case of tandemly arranged SDNs, larger deletions can be obtained (SDN1b). Furthermore, inversions (SDN1c) or translocations (SDN1d) can be generated by multiplexed SDN1 approaches (Hilscher *et al.*, 2016).
- (ii) SDN2 describes the use of a SDN with an additional donor DNA to introduce small mutations in a controlled manner. Here, a template mainly homologous to the target sequence is provided to be the substrate for HR-mediated DSB repair following the induction of one or two adjacent DSBs (Figure 4). This approach allows the introduction of small mutations that could also occur naturally, *per se*. Taking the size of plant genomes into account, small modifications up to 20 nucleotides can statistically be regarded as GE that resembles naturally occurring genome changes. Therefore, targeted genome modifications using ODM are also regarded comparable to SDN2.
- (iii) SDN3 describes the use of a SDN with an additional donor DNA to introduce large stretches of exogenous DNA at a pre-determined locus, adding or replacing genetic information. Mechanistically, this process relies on HR-mediated DSB repair like SDN2, and the discrimination is arbitrary as the size of the sequence inserted can vary significantly (Figure 4).

This classification attempts to differentiate between genome modifications that resemble natural or induced





**Figure 4.** Genome engineering via homologous recombination (HR).

Precisely targeted site directed nuclease (SDN)-mediated double-strand breaks (DSBs) can be exploited to achieve homology-directed repair (HDR) providing T-DNAs, DNA oligos, plasmids, viral replicons or donor DNA stably integrated in the plant genome (*in planta* GT) as repair templates. HR within the flanking sequences (red, blue) between donor and recipient yields the desired end product. In the absence of donor molecules with homology to the target, non-homologous end-joining-mediated DSB repair can lead to the incorporation of provided external sequences or result in targeted mutations by small insertions or deletions (not shown). SIR, short intergenic region; LIR, long intergenic region; Rep, replication initiator protein.

mutations from allele replacements or site-specific sequence integrations. While the genome modifications can be achieved via HR or NHEJ, and are therefore not mechanistically different, the (in)ability to discriminate between potential natural outcomes can provide a guideline for regulatory authorities to reshape their classification of GMOs and non-GMOs, providing scientists and agrobiotech companies with a clear view on what permissions are needed, for example for field trials or for bringing a new product to the market.

#### FUTURE PERSPECTIVES ON THE REGULATION AND ACCEPTANCE OF CROP PLANTS MODIFIED WITH NATURAL GENOME ENGINEERING METHODS

The new tools for site-directed GE pose a challenge for legislation authorities with regard to classification of events generated in this way as GMO or non-GMO, and consequently create the need for establishing clear guidelines for deregulation requirements. The current regulatory requirements vary between agencies, nations and regions of the world (McHughen and Smyth, 2008; Sprink *et al.*, 2016; Wolt *et al.*, 2016).

In the United States, the regulation of GMOs follows the Coordinated Framework for Regulation of Biotechnology (US Office of Science and Technology Policy (OSTP), 1986). Three agencies, the Department of Agriculture (USDA), the Food and Drug Administration (FDA) and the

Environmental Protection Agency (EPA) are responsible for the approval process for GE crops (McHughen and Smyth, 2008). In general, the approach in the United States is mainly product-oriented; for example, if a SDN-derived deletion or mutation (SDN1 and SDN2) is generated without leaving traces of foreign DNA, such as SDN-encoding sequences, the chances are high that the respective crop does not need to be regulated. However, if a SDN3 approach is followed and new sequences are permanently inserted into the plant genome, the USDA will regard the crop as one to be regulated. The USDA is the lead regulator of the three agencies and regulates GE crops to the extent that the derived plant may behave as a plant pest or weed. The FDA evaluation of the safety of GE crops, foods and feed derived thereof is based on compositional equivalence between the GE product and its non-transgenic comparator line, for example in regard to toxins, allergens or antinutrients (McHughen and Smyth, 2008). The focus of the EPA is on the regulation of pesticidal properties and thus also has its main attention on the final product.

The situation in Europe is completely different, as EU legislation defines GM crops specifically as 'an organism [...] in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination'. While recombinant nucleic acids involving artificial techniques are clearly included in regulation,

mutagenesis is explicitly excluded. Moreover, due to the adherence to the precautionary principle, product development in the EU has been hindered, causing retraction of the industry from the EU market (Hartung and Schiemann, 2014; Sprink *et al.*, 2016; Wolt *et al.*, 2016). Interestingly, at the end of 2015, the Swedish Board of Agriculture confirmed the interpretation that some plants in which the genome has been edited using the CRISPR/Cas9 technology do not fall under the European definition of GMOs.

While the regulatory principles in the United States seem to allow more flexibility than those in Europe, it is evident that even with the European process-based approach, potential risks may escape the regulators. Therefore, regulation should focus on the risks posed by the features of the product and not the breeding process (McHughen and Smyth, 2008).

The regulation of GE crops has been extensively discussed and reviewed by the scientific community and regulators (Breyer *et al.*, 2009; European Commission, JRC, 2011; EFSA Panel on Genetically Modified Organisms (GMO), 2012; Podevin *et al.*, 2012, 2013; Lusser and Davies, 2013; Pauwels *et al.*, 2014). Based on those considerations it can be concluded that the regulation procedure on GE crops should be mainly influenced by the outcome of the DNA repair processes involved as classified by the SDN1, SDN2 and SDN3 definitions of genome modification.

Editing plant genomes without the introduction of recombinant DNA may be beneficial to alleviate regulatory concerns towards genetically modified plants (Voytas and Gao, 2014; Kanchiswamy *et al.*, 2016). Therefore, DNA-free and virus-based GE tools may be of additional benefit in regions where legislation is more stringent, while leading to the same GE-outcome.

Using a *Nicotiana benthamiana* line overexpressing a Cas9 protein, it was shown that the gRNA can be delivered exploiting the RNA-based tobacco rattle virus (TRV) (Ali *et al.*, 2015). The advantage of this approach is that it allows multiplexing and the virus does not integrate into the plant genome.

In an elegant study (Stoddard *et al.*, 2016), it was shown that targeted mutagenesis in plant cells can be achieved by transformation of sequence-specific TALEN nuclease mRNA. While the mutation frequency at the chosen *ALS* gene locus of *N. benthamiana* was only 6%, as opposed to the 70.5% achieved by DNA-based delivery of the same nuclease, unintended sequence integration in the host genome could be reduced by a factor of three. Additionally, integrations were much smaller than in case of DNA-delivered nuclease. Different 5' and 3' untranslated regions from well-characterized *Arabidopsis thaliana* genes were applied in this study to enhance translation efficiency, subcellular localization and mRNA stability. Stoddard *et al.* (2016) concluded that the observed effect could be attributed to an increase in translational efficiency rather than mRNA

stability. Recently, it has been shown that transient delivery of DNA or RNA encoding for CRISPR/Cas9 allows for highly efficient and specific GE in *Triticum aestivum* and *Triticum durum* to obtain mutants without detectable transgenes (Zhang *et al.*, 2016).

Delivery of pre-assembled Cas9 protein–gRNA ribonucleoproteins (RNPs) into protoplasts of *Arabidopsis*, tobacco, lettuce and rice has been successfully tested and allowed regeneration of GE plants at frequencies of up to 46% (Woo *et al.*, 2015). GE frequencies varied between 8.4 and 44%. In a similar approach (Subburaj *et al.*, 2016) an average mutagenesis frequency of 11.5% in the *Petunia* nitrate reductase (NR) gene locus could be shown. Furthermore, biolistic delivery of pre-assembled RNPs into maize embryo cells and regeneration of plants with both mutated and edited alleles has recently been shown (Svitashev *et al.*, 2016).

In another study, a complete nucleic-acid free approach for plant GE was demonstrated (Luo *et al.*, 2015). Purified I-SceI ME was transfected in *Nicotiana tabacum* protoplasts and a successful GE frequency of 2.7% was established with a YFP reporter construct that was reconstituted via the SSA repair mechanism. Additionally, the authors were able to demonstrate that by transfection of much larger TALENs in *N. benthamiana*, GE frequencies of up to 1.4% could be obtained.

While the described DNA-free and virus-based GE techniques are very promising, it is important to note that independent of the discussed legislative obstacles, several genome edited crops obtained by SDN-mediated GE have been approved by the USDA under non-regulated conditions – most recently a polyphenol oxidase (*PPO*) knockout mutant of *Solanum tuberosum* generated by Calyxt (Table 1). In 2016, a DuPont/Pioneer generated corn plant with a CRISPR/Cas inactivated *WX1* gene, which encodes a granule-bound starch synthase catalyzing production of amylose, was approved as not to be regulated (Table 1). The resulting *waxy corn* phenotype was accomplished by deletion of the DNA sequence between two CRISPR/Cas-induced DSB sites, with the molecular scissors being delivered via biolistic transformation. While regular corn starch accounts for 70% of the kernel weight with an amylose/amylopectin ratio of 27%/73%, respectively, the *waxy corn* exclusively contains amylopectin which has superior physico-chemical properties and is widely used in the food and paper industry (Waltz, 2016a). Also in 2016, the USDA approved the white button mushroom *Agaricus bisporus* (Waltz, 2016b), in which transient expression of CRISPR/Cas in protoplasts led to small deletions in the *PPO* gene thus preventing browning during storage (Table 1).

As a result, it is evident that with the advent of the new SDN technologies, an increasing portfolio of gene and genome edited plant species have been and will be produced to obtain improved traits which are regarded as to not fall under GMO regulations, at least in the United States.

**Table 1** Overview of site directed nuclease (SDN)-engineered crops deemed by the US Department of Agriculture (USDA) not to be regulated. Over the last years, an increasing number of crop species have been genetically engineered with various SDN tools to obtain desirable phenotypes, mainly by gene knockouts. As most of these genome modifications fall under the SDN1 classification, the USDA decided that those products are not regarded to fall under the regulations at 7 CFR part 340 (§340.1)

Year	Company	Species	Tool	Classification	Method	Trait	USDA call	Literature
2016	SIMPLOT Plant Sciences	<i>Solanum tuberosum</i>	TALEN	SDN1	<i>Agrobacterium</i> -mediated transformation, regeneration of null segregant	<i>PPO</i> knockout, reduced bruising and browning	Not regulated	
2016	Calyxt	<i>Solanum tuberosum</i>	TALEN	SDN1	Transient PEG protoplast transformation, regeneration of null segregant	<i>PPO</i> knockout, reduced bruising and browning	Not regulated	Waltz (2015)
2016	DuPont/Pioneer	<i>Zea mays</i>	CRISPR-Cas	SDN1	Biolistic transformation and gene excision	<i>WX1</i> knockout, amylopectin corn	Not regulated	Waltz (2016a)
2016	Penn State University	<i>Agaricus bisporus</i>	CRISPR-Cas	SDN1	Transient PEG protoplast transformation, regeneration of null segregant	<i>PPO</i> knockout, anti-browning mushroom	Not regulated	Waltz (2016b)
2016	Calyxt	<i>Triticum aestivum</i>	TALEN	SDN1	Biolistic transformation of immature embryos	<i>MLO</i> knockout, improved powder mildew resistance	Not regulated	Wang <i>et al.</i> (2014)
2015	Agrivida	<i>Zea mays</i>	Meganuclease	SDN1	Unknown	Unknown gene alteration	Not regulated	
2015	Iowa State University	<i>Oryza sativa</i>	TALEN	SDN1	<i>Agrobacterium</i> -mediated transformation, regeneration of null segregant	<i>OsSWEET11</i> and <i>OsSWEET14</i> , disease resistance rice	Not regulated	Verdier <i>et al.</i> (2012), Li <i>et al.</i> (2013)
2015	Calyxt	<i>Glycine max</i>	TALEN	SDN1	Unknown transformation of soybean cotyledons and regeneration of calli	<i>FAD3A</i> and <i>FAD3B</i> knockout, low-linoleic acid soybean	Not regulated	
2015	Benson Hill	<i>Zea mays</i>	Meganuclease	SDN2/3	Biolistic transformation	BHB high yield maize, improved photosynthesis	Not regulated	
2015	Calyxt	<i>Glycine max</i>	TALEN	SDN1	Unknown transformation of soybean cotyledons and regeneration of calli	<i>FAD2A</i> and <i>FAD2B</i> knockout, high-oleic soybean	Not regulated	Haun <i>et al.</i> (2014)
2014	Collectis	<i>Solanum tuberosum</i>	TALEN	SDN1	Transient PEG protoplast transformation, regeneration of null segregant	Low-acrylamide potato	Not regulated	
2010	Dow AgroScience	<i>Zea mays</i>	ZFN	SDN1	Embryogenic cell culture transformation, plant regeneration from calli	Low-phytate corn	Not regulated	Shukla <i>et al.</i> (2009)

## FINAL CONSIDERATIONS

Considering the initially discussed processes of breeding and mutational breeding in comparison with the possibilities that arose with the advent of reprogrammable customized SDNs, it has become evident that plant biotechnology has reached a new era. While classical and mutational breeding were time-consuming and potential mutations occurred unnoticed elsewhere in the genome, the new technologies allow for fast and highly accurate GE, exploiting the natural DNA repair mechanisms of plants. Harnessing the knowledge of those pathways and the availability of the new biotechnological tools, it is now feasible to create precisely predictable genome modifications at affordable costs that are based on much better defined changes than classical breeding-induced mutations and are by no means different from naturally occurring varieties. Given those new possibilities of editing plant genomes, even without a permanent or even temporary presence of stably integrated DNA, regulatory burdens for commercialization of new agronomically relevant crops should be lowered and hopefully public acceptance enhanced. It is our strong opinion that the currently available tools will significantly boost the potential of plant biotechnology and in this way support the increasing nutritional needs of a steadily growing world population.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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