SCIENTIFIC OPINION

Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function[1]

EFSA Panel on Genetically Modified Organisms (GMO)[2, 3]

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ABSTRACT

The European Commission requested that the EFSA Panel on Genetically Modified Organisms deliver a scientific opinion related to risk assessment of plants developed using the zinc finger nuclease 3 technique (ZFN-3) which allows the integration of gene(s) in a predefined insertion site in the genome of the recipient species. Since other nucleases with a similar function to ZFN are considered in this opinion the term site-directed nuclease 3 (SDN-3) is used to describe the technique rather than ZFN-3 specifically. The EFSA GMO Panel considers that its guidance documents are applicable for the evaluation of food and feed products derived from plants developed using the SDN-3 technique and for performing an environmental risk assessment. However, on a case-by-case basis lesser amounts of event specific data may be needed for the risk assessment of plants developed using the SDN-3 technique. The EFSA GMO Panel compared the hazards associated with plants produced by the SDN-3 technique with those obtained by conventional plant breeding techniques and by currently used transgenesis. With respect to the genes introduced, the SDN-3 technique does not differ from transgenesis or from the other genetic modification techniques currently used, and can be used to introduce transgenes, intragenes or cisgenes. The main difference between the SDN-3 technique and transgenesis is that the insertion of DNA is targeted to a predefined region of the genome. Therefore, the SDN-3 technique can minimise hazards associated with the disruption of genes and/or regulatory elements in the recipient genome. Whilst the SDN-3 technique can induce off-target changes in the genome of the recipient plant these would be fewer than those occurring with most mutagenesis techniques. Furthermore, where such changes occur they would be of the same types as those produced by conventional breeding techniques.

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KEY WORDS

TALEN, meganuclease, ZFN, genome editing, gene targeting, transgenic, site-directed nucleases

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SUMMARY

Following a request from the European Commission (EC), the EFSA Panel on Genetically Modified Organisms (GMO) was requested to deliver a scientific opinion on plants developed through eight techniques in terms of the risks they might pose and the applicability of the existing guidance documents for their risk assessment. The mandate included two specific questions:

1. Determine whether there is a need for new guidance or whether the existing guidance on risk assessment should be updated or further elaborated, in anticipation of the placing of products on the market through the application of the listed techniques.

2. What are the risks in terms of impact on humans, animals and the environment that the eight techniques listed could pose, irrespective of whether or not they fall under the GMO legislation? This latter request should consider the most recent scientific literature and knowledge of plant breeding experts and compare plants obtained by these new techniques with plants obtained by conventional plant breeding techniques and secondly with plants obtained with currently used genetic modification techniques.

After delivery of an opinion on cisgenesis/intragenesis the European Commission requested that the EFSA GMO Panel deliver a scientific opinion related to risk assessment of plants developed using the zinc finger nuclease 3 technique (ZFN-3), which allows the integration of gene(s) into a predefined insertion site in the genome of the recipient species. Since other nucleases with a similar function to ZFN are considered in this opinion the term site-directed nuclease 3 (SDN-3) is used to describe the technique rather than ZFN-3 specifically.

While addressing question two of the mandate, the EFSA GMO Panel compared the hazards associated with plants produced by the SDN-3 technique with those associated with plants obtained by conventional plant breeding techniques and by currently used transgenesis. With respect to the genes introduced, the SDN-3 technique does not differ from transgenesis or from the other genetic modification techniques currently used with respect to any hazards associated with the introduced genes. The SDN-3 technique can be used to introduce transgenes, intragenes or cisgenes.

The main difference between the SDN-3 technique and transgenesis is that the insertion of DNA is targeted to a predefined region of the genome. Therefore, the SDN-3 technique can minimise hazards associated with the disruption of genes and/or regulatory elements in the recipient genome. Whilst the SDN-3 technique can induce off-target changes in the genome of the recipient plant these would be fewer than those occurring with most mutagenesis techniques used in conventional breeding. Furthermore, where such changes occur they would be of the same types as those produced by conventional breeding techniques.

In response to question one of the mandate, the EFSA GMO Panel considers that the *Guidance for risk assessment of food and feed from genetically modified plants* (EFSA, 2011) and the *Guidance on the environmental risk assessment of genetically modified plants* (EFSA, 2010) are applicable for the evaluation of food and feed products derived from plants developed using the SDN-3 technique and for performing an environmental risk assessment. However, on a case-by-case basis lesser amounts of event-specific data may be needed for the risk assessment of plants developed using the SDN-3 technique. There is therefore a need for flexibility in the data requirements for risk assessments.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

New breeding and genetic modification techniques have evolved at a rapid pace since the introduction of the legislation in 1990 with the result that in some instances it is unclear whether they give rise to GMOs pursuant to EU legislation. This is especially relevant for plant breeding as some of these breeding and genetic modification techniques have been subject to field trials in the EU and a number of them are now approaching commercialisation.

At the request of the Competent Authorities (CA) under Directive 2001/18/EC, a New Techniques Working Group (NTWG) was established in October 2007 to analyse a non-exhaustive list of techniques for which it is unclear whether they would result in a genetically modified organism or a genetically modified micro-organism as defined under Directive 2001/18/EC or Directive 2009/41/EC respectively.

An initial list of eight techniques was proposed by the CA for consideration by the NTWG. At the time of requesting this opinion the final report from December 2011 was provided for information. The Terms of Reference as endorsed by the CA state that "the findings of the WG may be referred to EFSA for opinion".

BACKGROUND AS PROVIDED BY EFSA

Following a request of the European Commission (DG SANCO Ares(2011)201516 – 23/02/2011), EFSA set up a working group of the EFSA GMO Panel on new techniques in April 2011. The mandate concerned eight new techniques: (1) zinc finger nuclease technique (ZFN), comprising ZFN-1, ZFN-2 and ZFN-3; (2) oligonucleotide-directed mutagenesis; (3) cisgenesis; (4) RNA-dependent DNA methylation via RNAi/siRNA; (5) grafting; (6) reverse breeding; (7) agro-infiltration; (8) synthetic biology. The European Commission proposed a phased consideration of the new techniques starting with cisgenesis and intragenesis. The EFSA GMO Panel adopted an opinion on cisgenesis and intragenesis and this was published on 16 February 2012. Subsequently, the European Commission requested EFSA to address zinc finger nuclease technique 3 (ZFN-3), as described in the Member State experts "New Techniques Working Group Final Report" (December 2011), in its letter dated 11 May 2012 (DG SANCO Ares(2012)573179).

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

Against this background, the Commission would like to ask EFSA to address the following considerations in separate opinions per technique or for groups of techniques as appropriate:

1. Determine whether there is a need for new guidance or whether the existing guidance on risk assessment should be updated or further elaborated, in anticipation of the placing of products on the market through the application of the listed techniques.

2. What are the risks in terms of impact on humans, animals and the environment that the eight techniques listed could pose, irrespective of whether or not they fall under the GMO legislation? This latter request should consider the most recent scientific literature and knowledge of plant breeding experts and compare plants obtained by these new techniques with plants obtained by conventional plant breeding techniques and secondly with plants obtained with currently used genetic modification techniques.

ASSESSMENT

1. INTRODUCTION

In dealing with the request from the European Commission (EC) the EFSA GMO Panel initially focused on plants developed by the zinc finger nuclease 3 technique (ZFN-3). The ZFN-3 technique has been defined by the working group of EU Member States' experts on new techniques as follows:

Zinc Finger Nuclease-3 technique *(ZFN-3) targets delivery of transgenes (insertions) by homologous recombination. DNA fragments or gene cassettes up to several kilo base pairs (kbp) in length can be inserted precisely to a desired site in the genome or a gene. In practice, a recombinant DNA molecule is constructed in which the DNA fragment or the gene cassette of interest (donor DNA) is sandwiched between stretches of DNA that are homologous with the DNA sequences flanking the DSB* (double stranded break) *site. Donor DNA can come from any species and it is delivered to the cell, along with the ZFN, and it is targeted to the desired site of the genome and inserted into the DSB site.*

For completeness and clarity, and to place the ZFN-3 technique in a broader context, it is important that the associated techniques, ZFN-1 and ZFN-2, are also briefly described. The ZFN-1 and ZFN-2 techniques have been defined by the New Techniques Working Group (NTWG) of EU Member States as follows:

Zinc Finger Nuclease-1 technique *(ZFN-1) generates site-specific random mutations (changes of single base pairs, short deletions and insertions) by non-homologous end-joining.*
During ZFN-1, no repair template is provided to the cells together with the ZFN. The DSB is repaired by non-homologous end-joining which is a natural DNA-break repair mechanism in the cell. This often (though not always) results in a single or a few base substitutions or small localized deletions or insertions. In the case of insertions, the inserted material is derived from the organism's own genome i.e. it is not exogenous. The DNA end (from the strand break) may also become joined to a completely unrelated site, which results in chromosomal translocation.

Zinc Finger Nuclease-2 technique *(ZFN-2) generates site-specific desired point mutation by DNA repair processes through homologous recombination (specific nucleotide substitutions of a single or a few nucleotides or small insertions or deletions).*
During ZFN-2, a continuous stretch of DNA is delivered to the cells simultaneously with the ZFN. This template DNA is homologous to the targeted area, spanning a few kilo base pairs (kbp), and overlaps the region of the DSB. The template DNA contains the specific base pair alteration(s) to be introduced into the target DNA or chromosome. The exogenous repair DNA competes with the sister chromatids as a repair template and - with a low frequency - leads to replacement of the original nucleotide sequence. In most studies, the aim has been to replace one or a few bp. There are indications that efficiency of repair decreases where the number of mismatches increases in the template DNA with increasing distance from the DSB. The result is thus comparable with some other site-specific mutagenesis methods.

In dealing with the requests from the EC the EFSA GMO Panel has, in this opinion, focused on the hazards associated with plants developed by the ZFN-3 technique compared with plants developed by conventional breeding or currently used genetic modification techniques as defined by Directive 2001/18/EC. Within this mandate the EFSA GMO Panel has focussed on currently used transgenesis[4] as the genetic modification technique used in comparisons with the ZFN-3 technique. As requested by the EC, the Panel considered the most recent and relevant scientific literature together with the expertise and experience of plant breeding experts. The Panel then considered the applicability of its current guidance documents on the risk assessment of genetically modified (GM) plants to the assessment of plants developed by the ZFN-3 technique.

[4] As defined in the glossary

With regard to comparisons between the safety of plants developed by the ZFN-3 technique and those developed by conventional breeding and genetic modification techniques currently used, the EFSA GMO Panel has used the following approach (see sections 2–4):

i) An assessment of conventional breeding approaches as the baseline for all comparisons. This includes a description of the conventional breeding approaches most closely related to breeding using the ZFN-3 technique.

ii) A comparison of the sources of hazards arising from conventional breeding and breeding using the ZFN-3 technique and genetic modification techniques currently used in transgenesis.

With regard to the applicability of current EFSA guidance documents for the risk assessment of transgenic plants to the assessment of plants developed by ZFN-3, the EFSA GMO Panel has evaluated all components of the molecular characterisation, food/feed and environmental safety evaluations with regard to the question whether the existing guidance on risk assessment should be updated or further elaborated (see section 5).

Since the identification of the non-exhaustive list of techniques by the Competent Authorities of Member States in 2007, there have been considerable developments not only in ZFN techniques (e.g. other applications) but also in the use of other enzymes with similar function (so called site-directed nucleases [SDNs]).

2. TECHNICAL DESCRIPTION

The site-directed nuclease technologies have evolved rapidly and recently, both in terms of their applications and the enzymes used. Therefore, the EFSA GMO Panel has broadened the analysis of the technologies accordingly.

Currently ZFN, transcription activator like effector nuclease (TALEN) and meganuclease (MN) approaches have been described and shown to act in a very similar manner (Tzfira et al., 2012). Thus, although the design of these approaches will differ they can all be used to develop the same plant traits and products. In the future additional nucleases might be developed. Therefore, and as agreed by the EC, this opinion has been developed to include all site-directed nuclease techniques (SDN) which deliver the genetic modifications associated with the ZFN-3 technique. This opinion refers to these generically as the SDN-3 technique. Similarly, genetic modifications associated with the ZFN-1 and -2 techniques can be made using TALEN and MN and therefore ZFN-1 can be categorised as an SDN-1 technique and ZFN-2 as an SDN-2 technique.

In SDN-1 applications, only the SDNs are introduced (stably or transiently), generating site-specific mutations by non-homologous end-joining (NHEJ) (see Fig. 1). In SDN-2 applications, homologous repair DNA (donor DNA) is introduced together with the SDN complex to create specific nucleotide sequence changes by homologous recombination (HR). The SDN-2 technique can result in minor or more substantial changes to the nucleotide sequences of the target gene. In the SDN-3 technique a large stretch of donor DNA (up to several kilobases) is introduced together with the SDN complex to target DNA insertion into a predefined genomic locus. The predefined locus may or may not have extensive similarity to the DNA to be inserted. The insertion can take place either by HR or by NHEJ. In the case of insertion by means of NHEJ, the technique is denominated the SDN-3–NHEJ technique.

Although the SDN-1, -2 and -3 approaches all target a specific locus in the genome and use a nuclease to induce breaks in DNA, the three SDN techniques trigger different repair outcomes, the intended changes range from point mutations to large insertions and deletions (Fig. 1). As the mandate given by the EC specifically requested an opinion focusing on the ZDN-3 technique, only SDN-3 technique will be discussed in detail.

In order to be considered a successful SDN-3 strategy, plants derived using the SDN-3 technique which are intended for placing on the market should:

- not contain any part of the SDN-cassette or any donor DNA integrated at non-targeted loci,
- contain only the intended sequences at the target site, and
- retain the original structure of the insertion locus.

Different strategies (including backcrossing) can be used to obtain a final product lacking sequences other than the intended sequences at the target site.

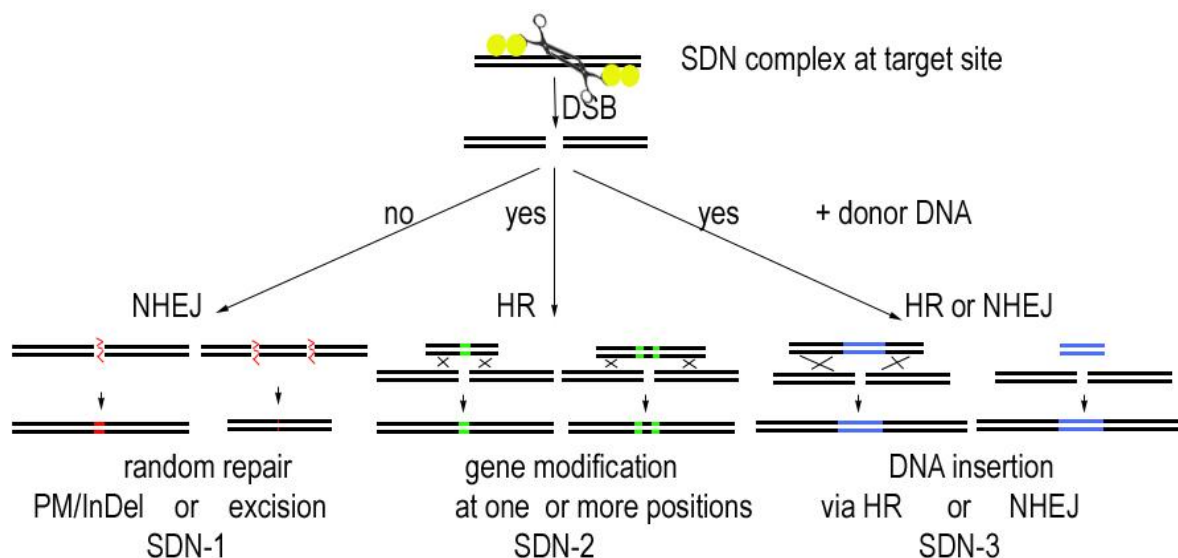

Figure 1: Intended repair outcomes of double-strand breaks (DSBs) induced by site-directed nuclease (SDN). An SDN complex is shown at the top in association with the target sequence. The repair can be achieved by non-homologous end-joining (NHEJ) or homologous recombination (HR) using the donor DNA. In the SDN-1 technique, double-strand DNA breaks result in site-specific random point mutations (PM) and short insertions/deletions (indel) or in excision. In the SDN-2 technique, homologous donor DNA is used to induce specific nucleotide sequence changes by HR. In the SDN-3 technique, DNA may be integrated in the plant genome using either NHEJ or HR.

To achieve the required DNA changes, SDN technique utilises three different steps:

i. DNA binding, using an intrinsic protein domain (as for MN) or an engineered zinc finger (ZF) or transcription activator-like effector (TALE) DNA-binding domain. In all SDNs, such domains are responsible for binding to a specific DNA sequence while a second domain is responsible for DNA cleavage at the binding site.

ii. DNA cleavage, which is achieved by a nuclease domain of the same protein (MN) or an artificially coupled restriction endonuclease (ZFN/TALEN).

iii. DNA repair, which relies on the host DNA repair machinery and can proceed through NHEJ or homologous recombination HR (see section 2.2). NHEJ is the preferred DNA repair mechanism in somatic cells of eukaryotes (exceptions are the yeast *Saccharomyces cerevisiae* and in higher eukaryotes the moss *Physcomitrella patens*) and depends solely on the host's cellular DNA repair machinery (such as in SDN-1). HR is essential to repair DSBs which occur during meiotic recombination and replication. Although HR is not frequently used for DNA repair in somatic cells, it can be modulated to a certain extent by the simultaneous addition of homologous DNA sequences during the DNA repair of DSBs to shift the balance between NHEJ and HR (SDN-2 and -3). The introduction at the targeted locus of a single-

strand break (SSB) rather than a DSB can also enforce the DNA repair via HR. Recently, targeted SSBs have been obtained using a nicking enzyme (ZF nickase) (McConnell Smith et al., 2009; Gonçalves et al., 2012; Ramirez et al., 2012; Wang et al., 2012). ZF-nickases can stimulate HR at their nicking site in human cells (albeit at a lower frequency than the ZFNs from which they were derived) and appear to result in greatly reduced levels of NHEJ at their target nicking site or at off-target sites (Metzger et al., 2011).

The different types of nucleases have advantages and disadvantages (Curtin et al., 2012). The small size of MNs makes them easier to deliver and the high specificity creates less damage to the cell in terms of a lower frequency of DSBs at off-target DNA sequences. However, their structure, as well as the complex array of protein/DNA-specific interactions they establish, makes them less flexible and more difficult to engineer. On the other hand, the modular nature of the DNA-binding motifs of both ZFNs and TALENs makes them highly flexible and allows the engineering of DNA-binding modules for virtually any sequence of interest. However, a reduced specificity has been observed with ZFNs compared with MNs, leading to significant off-target DSBs on DNA and higher levels of cellular damage.

2.1. Site-directed nucleases (SDNs)

2.1.1. *Nucleases fused with DNA-binding domains: ZFNs and TALENs*

ZFNs and TALENs are created by fusing either a zinc finger (ZF) DNA-binding domain or a transcription activator-like (TAL) effector DNA-binding domain, respectively, to the DNA-cleavage domain of the *Fok*I endonuclease (Fig. 2).

ZF proteins are commonly found in eukaryotes (Laity et al., 2001). The DNA-binding domains of ZF proteins typically contain three individual ZFs whereby each finger recognises 3 bp (Kim et al., 1996). The ZFs can be linked together in a peptide designed to bind a predetermined DNA site. The DNA-binding domains of individual ZFNs typically contain between three and six individual ZF repeats and thus recognise between 9 and 18 nucleotides (nt).

The TAL effectors are transcription factors produced by the plant pathogen *Xanthomonas*. They are injected into plant cells via the bacterial type III secretion system, imported into the plant cell nucleus, and targeted to specific elements in plant gene promoters in order to activate the expression of plant genes that aid bacterial infection (Kay et al., 2007; Römer et al., 2007; Bogdanove et al., 2010). The targeting/DNA-binding domain of TAL effectors contains highly conserved 33-35 amino acid sequence repeats. Polymorphism among the repeats is almost exclusively localised to a pair of residues at positions 12 and 13. These two locations, termed the repeat-variable di-residue (RVD), are highly variable, and determine the specificity of the nucleotide binding of each repeat (Boch and Bonas, 2010). This simple relationship, whereby each repeat binds a specific nucleotide, has facilitated the engineering of specific DNA-binding domains by selecting a combination of repeat segments containing the appropriate RVD (Boch et al., 2009; Moscou and Bogdanove, 2009). The number of repeats and the sequence of the RVD determine the length and sequence of the target sequence that will be recognised. DNA-binding domains of individual TALENs typically contain between 12 and 30 RVDs and can thus recognise between 12 and 30 nt.

The source of the DNA cleavage domain of the fusion protein is usually from the *Fok*I enzyme from *Flavobacterium okeanokoites*. *Fok*I is a bacterial type IIS restriction endonuclease which cleaves 9/13 nt downstream of the recognition site (Szybalski et al., 1991; Bitinaite et al., 1998). The *Fok*I functions as a dimer, and is most efficient when the two binding sites are separated by a spacer. For these reasons, both ZFNs and TALENs that contain the DNA-cleavage domain of *Fok*I are designed in pairs that bind opposing DNA target sites separated by a spacer sequence.

Significant off-target activity has been reported with ZFNs as a result of DNA cleavage at unintended locations. This is the main reason for the cellular damage observed when using ZFNs (Cornu et al.,

2008; Gabriel et al., 2011; Pattanayak et al., 2011). Therefore, in principle, every ZFN should be tested *in vitro* for off-target site activity, although this approach does not always reflect the degree of off-target activity *in vivo* (Cornu et al., 2008; Pattanayak et al., 2011). Four major determinants of ZFN and TALEN activity and specificity have been examined to date: the DNA-binding domain (Kim et al., 2010; Streubel et al., 2012); the nuclease domain (Miller et al., 2007; Szczepek et al., 2007); the linker sequence connecting the two (Händel et al., 2009); and the way the SDN is expressed (stable [inducible or tissue specific] or transient). Different strategies exist and are under development to increase the specificity and decrease DNA damage arising from the use of both ZFNs and TALENs by adjusting the DNA and/or nuclease domain.

The DNA-binding domain

Most of the specificity of SDNs is usually provided by the DNA-binding domain as the DNA-cleavage domain is often unspecific. SDNs that do not contain a DNA-binding domain with sufficient affinity and specificity for the recognition site either will not find the DNA target or will bind and cleave similar sequences in the genome and therefore cause toxicity.

Several methods have been described to generate ZF-based DNA-binding domains (Isalan and Choo, 2001; Mandell and Barbas, 2006; Maeder et al., 2008; Urnov et al., 2010). It has become clear that, independent of the strategy used to generate the ZFN, optimisation of affinity as well as specificity is required (Cornu et al., 2008). One of the main reasons for low specificity is finger–finger incompatibility due to context-dependent effects. Assembly methods have been developed to circumvent this problem include finger stitching and base skipping.

To date four RVDs have been mainly used, but analysis has shown that other RVDs can be used to increase activity and specificity (Boch et al., 2009; Morbitzer et al., 2010; Huang et al., 2011; Miller et al., 2011; Streubel et al., 2012).

The cleavage domain

In the case of SDNs fused to wild-type *Fok*I cleavage domains, SDN homodimers may also form which limit their efficacy and which induce off-target DNA cleavage. Therefore, *Fok*I cleavage domain variants that preferentially heterodimerise have been developed, and the corresponding engineered SDNs have increased specificity (Miller et al., 2007; Szczepek et al., 2007; Sollu et al., 2010; Doyon et al., 2011). An adjusted strategy is to create a single-chain ZFN (Minczuk et al., 2008; Mino et al., 2009). Single-chain ZFNs seem to be more specific than classical dimeric ZFNs and have been shown to be capable of discrimination between DNA differing in sequence by only 1 nt (Mino et al., 2009).

A new strategy to increase the specificity even further is to combine a ZF or a catalytically inactive MN with the restriction endonuclease *Pvu*II (Fonfara et al., 2012; Schierling et al., 2012), instead of *Fok*I. This results in two levels of cleavage site specificity: the ZF or MN must recognise its specific DNA-binding site and in doing so cleavage will occur only if the recognition sequence for *Pvu*II is also present at this site. The usage of other nuclease domains recognising 2 to 6 specific nucleotides are under development.

An additional new development is the use of nickases that will produce a SSB rather than a DSB (Ramirez et al., 2012; Wang et al., 2012). It has been shown in animal systems, that nickases can efficiently initiate targeted gene correction because nicks induce less genome instability than DSBs as they are mainly repaired by either direct ligation or homology-directed repair (Davis and Maizels, 2011; Metzger et al., 2011).

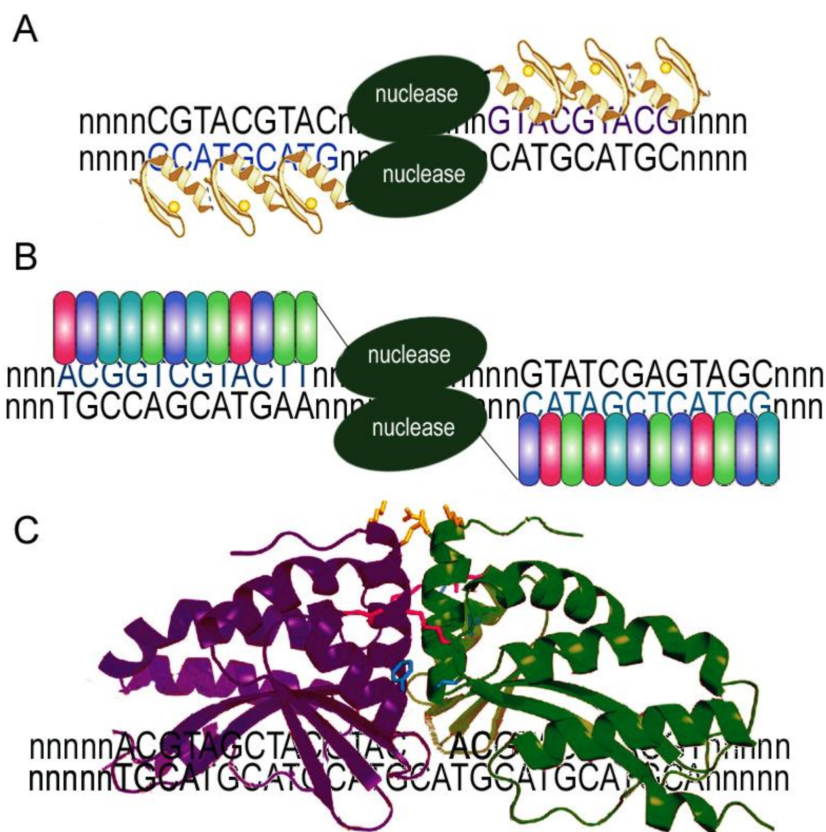

Figure 2: Structures of currently used site-directed nucleases (SDNs) at their target locus. The DNA sequence specifically recognised by the SDN is indicated by A, T, C and G while, the sequences not recognised by the SDN proteins are indicated by n. (A) A pair of ZFNs bound to DNA. The ZFs are shown in yellow and the *Fok*I cleavage domains in dark green. (B) A pair of TALENs bound to DNA. The central repeat domains are shown as coloured bars with the diversity in the repeat-variable di-residue shown in a different colour. The *Fok*I cleavage domains are shown in dark green. (C) The meganuclease complex showing the α-helices, β-strands, and loops (modified from Fajardo-Sanchez et al., 2008).

2.1.2. *Meganuclease*

Meganucleases (MNs) are naturally occurring endodeoxyribonucleases characterised by a large recognition site (DNA sequences of 14–40 bp). MNs are considered the most specific naturally occurring restriction enzymes. They are found in a wide range of organisms (e.g. archaea, bacteria, fungi, yeast, algae and some plants) and can be expressed in different subcellular compartments including the nucleus, mitochondria and plastids. These proteins either are encoded by introns of a gene or are components of another protein. After activation by splicing, MNs catalyse the copying of their own coding sequence into the allelic gene, which did not previously contain this sequence.

The best-studied MNs and most widely used include I-*Sce*I (discovered in the mitochondria of baker's yeast, *S. cerevisiae*), I-*Cre*I (from the chloroplasts of the green algae *Chlamydomonas reinhardtii*) and I-*Dmo*I (from the archaebacterium *Desulfurococcus mobilis*) (Pâques and Duchateau, 2007). These enzymes function as homodimers (e.g. I-*Cre*I) or internally symmetrical monomers (I-*Sce*I). The DNA-binding site, which contains the catalytic domain, is composed of two parts on either side of the cutting point (Stoddard, 2011) (Fig. 2).

Genetic recombinations that can be induced by using naturally occurring MNs are limited by the repertoire of MNs available. Despite the occurrence of hundreds of MNs, and even though MN can

tolerate minor variations in its recognition site, the probability of finding a naturally-occurring MN able to cut a given gene at the desired location is extremely low.

Tailor-made MNs have been designed using two approaches. The first one involves modifying the specificity of the MN by introducing a small number of variations in the amino acid sequence of the recognition site (Seligman et al., 2002; Rosen et al., 2006). The second approach is based on the association or fusion of protein domains from different enzymes (Grizot et al., 2010). This approach makes it possible to develop chimeric MNs with a new recognition site composed of a half-site of protein A and a half-site of protein B. These two approaches can be combined to increase the possibility of developing new enzymes while maintaining a high degree of efficacy and specificity.

2.1.3. *Double-strand break repair in plants*

Plant cells are subject to DNA damage, including DSBs, arising from intrinsic cellular processes (e.g. recombination during meiosis) and external factors such as ionising radiation and chemical mutagens (see section 3.2).

Two mechanisms of DSB repair can be used by the cell, the NHEJ pathway or HR-based repair. In eukaryotic somatic cells, including plant cells, the NHEJ pathway is generally preferred (Waterworth et al., 2011). The NHEJ repair simply re-joins the broken DNA ends without the use of a homologous template. This can result in unfaithful repair, creating nucleotide insertions and/or deletions (indels). The products of NHEJ have been studied in plants using extrachromosomal rejoining assays, repair of endonuclease-induced DSBs and analysis of chromosome fusions in telomerase mutants (Salomon and Puchta, 1998; Gorbunova and Levy, 1999; Heacock et al., 2004). These studies indicate frequent utilisation of microhomologies at the break site, deletions and appearance of insertions of “filler” DNA indicative of a DNA synthesis-dependent repair process whereby a single-stranded DNA end primes DNA synthesis using an ectopic site of the genome as a template.

HR plays a major role during meiosis. During HR, nucleotide sequences are exchanged between two very similar or identical DNA molecules.

2.2. The SDN-3 technique in plants

The SDN-3 technique targets DNA insertion into a predefined genomic locus. This locus may or may not have extensive similarity to the DNA to be inserted as the purpose of SDN-3 technique is to allow insertions or exchanges of entire genes or any other DNA sequence at a specific locus. Thus, SDN-3 technique can be used for transgenesis as well as for cisgenesis and intragenesis. The induction of a DSB at a particular locus with an SDN greatly increases the targeted integration of DNA, which otherwise would integrate randomly into naturally induced chromosome breaks. Therefore, the use of SDNs makes it possible to insert DNA at a specific locus in the plant genome. The integration of the DNA can be mediated by HR or by NHEJ (the latter is designated SDN-3–NHEJ technique), depending on the presence or not of sequence similarity between the DNA to be inserted and the target locus.

2.2.1. *Donor DNA delivery mechanism and expression of SDNs*

The gene encoding the SDN (ZFN, TALEN or MN) that will recognise the target locus, and the donor DNA bearing the transgene flanked by DNA stretches with homology to this locus, can be delivered into the plant cell via various methods. The SDN-encoding gene can be expressed transiently or can be stably integrated into the host genome.

Transient expression of SDN: To date, examples of co-delivery of the SDN and donor DNA for transient expression include electroporation of tobacco protoplasts (Wright et al., 2005), *Agrobacterium*-mediated transformation of tobacco protoplasts and tobacco leaf discs (Cai et al., 2009), biolistics or *Agrobacterium*-mediated transformation of maize suspension cells (D'Halluin et al., 2008), and whisker-mediated transformation of maize embryos (Shukla et al., 2009).

Stable expression of SDN: In this case, a plant constitutively expressing the SDN is first developed. This SDN-expressing plant is then transiently transformed with the donor DNA (e.g. via floral-dip mediated transformation as reported for *Arabidopsis* [de Pater et al., 2009]). The excision and reinsertion "*in planta*" into a targeted locus of a transgene already present in the plant has been achieved in *Arabidopsis* (Fauser et al., 2012). Once the targeted integration of the transgene has been achieved, the introduced SDN gene and the donor DNA at non-targeted loci can be removed by segregation to generate plants containing only the targeted integration of the transgene but no other foreign DNA.

2.2.2. ***Targeted integration of donor DNA via homologous recombination***

The use of constructs in which the sequence to be introduced, which can be several kbp long, is flanked by sequences identical to the DNA sequences at the cleavage site of a target locus in the recipient facilitates targeted integration by HR. However, in the absence of an SDN enzyme, the frequency of gene targeting (the ratio of targeted, via HR, versus random, via NHEJ, integration of the transgene) is very low (close to 10^{-5}), and is independent of the plant species and the transformation method used (Puchta, 2003). The first evidence that a break can increase the frequency of gene targeting, i.e. the level of HR- versus NHEJ-mediated integration, was shown in tobacco, in which cleavage of an artificial chromosomal target by the I-*Sce*I MN caused a 10 to 100-fold increase in the targeted integration (via HR) of the transforming construct (average ratio of targeted versus random integration of the transgene in the presence of I-*Sce*I MN is 10^{-3}) (Puchta et al., 1996). For HR based approaches, the resulting sequence at the insertion locus can be predicted based on prior knowledge of the DNA regions capable of HR.

More recently, the use of SDNs to facilitate the targeted integration of transgenes into an artificial locus has been reported in tobacco (Wright et al., 2005; Cai et al., 2009), maize (D'Halluin et al., 2008) and *Arabidopsis* (de Pater et al., 2009). In tobacco, Wright et al. (2005) co-transformed protoplasts from different lines containing an artificial target locus with the DNA encoding the ZFN and with a transgene flanked by more than 1 kbp upstream and almost 3 kbp downstream sequences identical to the artificial chromosome target. In this experiment more than 10 % of the transformed protoplasts contained the transgene at the target locus regardless of the chromosomal position of the target. In maize, co-delivery of the transgene and the DNA encoding the I-*Sce*I MN was achieved using either biolistics or *Agrobacterium*-mediated transformation (D'Halluin et al., 2008) and the presence of the I-*Sce*I MN led to an estimated 14 to 30 000-fold increase in the frequency of targeted versus random integration of the transgene. The variation in increase of gene targeting efficiency was dependent on the technique of DNA delivery used and on the artificial target site selected. In the case of *Arabidopsis*, the line containing the artificial chromosome target (T-DNA containing a GFP/GUS reporter gene including a ZFN target sequence) also contained a constitutive ZFN expression cassette. Floral dip transformation of this line with a T-DNA construct with homology to the target locus led to an estimated 10 to 1 000-fold increase in gene targeting efficiency (de Pater et al., 2009). Fauser et al. (2012) used a combination of three stably transformed constructs: one corresponding to the target locus, one to the donor locus (containing a partial GUS expression cassette transgene), both bearing I-*Sce*I recognition sites, and the third one corresponding to a cassette for constitutive I-*Sce*I MN expression. The presence of these three constructs in the same plant leads to the concomitant release of the donor DNA and cleavage of the targeted locus and results in the HR-driven targeted integration of the transgene. The analysis of the progeny of this plant led to the identification of targeted events in approximately 1 % of seeds analysed, depending on the combination of donor target.

Examples of use of the SDN-3 technique for targeted integration of a transgene into a natural plant locus are rare. However, targeted gene insertion into a natural chromosomal target site has been reported in maize (Shukla et al., 2009) and tobacco (Cai et al., 2009). In maize, the *pat* gene (which encodes phosphinothricin acetyltransferase and confers tolerance to glufosinate-ammonium based herbicides) was inserted into the *ZmIPK1* gene. This gene codes for an enzyme involved in the final step in the phytate biosynthesis pathway. Constructs containing a *pat* gene flanked by sequences (815 nt) identical to the *ZmIPK1* gene were co-transformed with the construct encoding the ZFN targeted

to the *ZmIPK1* gene in maize cells (Shukla et al., 2009). In tobacco, the stress-related *Chn50* endochitinase gene is highly expressed in the stationary-phase of suspension culture cells and targeting this site for transgene integration may be useful for recombinant protein production. In order to test the efficiency of the SDN-3 technique in targeting this locus, a gene conferring herbicide tolerance, driven by a constitutive promoter flanked on each side by 750 nt of the *Chn50* gene sequence, was co-delivered with a ZFN expression cassette into tobacco cells and leaf discs via *Agrobacterium* (Cai et al., 2009). Five per cent of the isolates generated from the suspension-cultured cells and 10 % of the plant events regenerated from leaf discs were targeted to the expected site.

2.2.3. *Targeted integration of donor DNA via non-homologous end-joining (SDN-3–NHEJ)*

Whilst the induction of a DSB with an SDN can increase the integration via HR of a transgene into a defined locus, DNA repair of the induced break can also be achieved by NHEJ. Targeted integration of a gene into a predefined site by NHEJ has been described by Tzifra et al. (2003), who transformed a tobacco line containing an artificial target locus bearing an I-*Sce*I recognition site with a T-DNA showing no homology to the target site. NHEJ-based gene targeting of this transgene was increased by a factor of at least 25 800. In contrast to integration by HR, in which the resulting sequence at the insertion locus can be predicted ("faithful integration"), the targeted integration of the transgene by NHEJ is usually accompanied by deletion or insertion of DNA at the targeted locus. However, strategies are under development to shift the balance towards faithful integration using the SDN-3–NHEJ technique by cleaving also the donor DNA. The main advantage of the SDN-3–NHEJ technique is the possibility of introducing large DNA sequences.

2.3. Conclusion

The SDN-3 technique targets insertion of DNA sequences into a predefined region of the genome, in contrast to commonly used methods for genetic modification, which result in random integration into the plant genome. The donor DNA sequence to be integrated by the SDN-3 technique may or may not contain similarity to the locus targeted for the insertion and the insertion can be achieved by either HR or NHEJ. The SDN-3 technique makes use of the same delivery methods as transgenesis and can achieve the desired outcome using either stable transformation or transient expression systems.

3. Conventional plant breeding techniques relevant for a comparison with SDN-3 technique

Within the context of this document, conventional plant breeding is defined as methods used by plant breeders for the improvement of commercial varieties and where the resulting plants/varieties are not covered by the legal definitions of genetic modification (Directive 2001/18/EC). Breeding for the improvement of commercial plant varieties involves selection of plants carrying the desired traits acting upon existing variation and/or newly created variation. For any given plant species, the genetic variants required for the development of new, advanced, varieties may already exist within the current gene pool of commercial lines. In other cases, the plant breeder needs to access genes from a wider pool to obtain the required traits of interest, e.g. resistance to evolving pests and pathogens. The sources of genes available for conventional plant breeding are referred to as the "breeders' gene pool". Conventional breeders distinguish between primary, secondary and tertiary gene pools (EFSA, 2012). It is well known that the genetic variability present within the gene pool is the result of different mechanisms acting on plant genomes (Friedburg et al., 2006; Morgante et al., 2007). Methods used to introduce genetic variation are diverse, ranging from approaches to manipulate whole genomes (polyploidisation) to the introduction of genes from wild relatives of cultivated plants (wide crosses, followed by introgression and translocation breeding). In addition, plant breeders can use physical and chemical mutagens to produce mutations in plant genomes. In general, all breeding approaches require subsequent selection steps to maximise the benefits and minimise undesirable consequences. Selection techniques range from phenotyping for agronomic performance to more sophisticated techniques such as marker-assisted selection. As genome sequencing leads to the discovery of genes of importance for crop improvement, allele mining using modern molecular

breeding techniques is likely to result in direct selection for the desired alleles. Before commercialisation, all new varieties have to be shown to be distinct, uniform and stable (DUS), for National Listing, Plant Breeders' Rights and Multiplication (Certification).

Conventional breeding methods include a wide range of techniques (van der Wiel et al., 2010). The EFSA GMO Panel considers the following techniques relevant for a comparison with plants developed by the SDN-3 technique: sexual crosses, bridge crosses, embryo rescue, somatic hybridisation, translocation breeding and mutation breeding. Additional information on all these techniques except for mutagenesis can be found in the EFSA's opinion on cisgenesis and intragenesis (EFSA, 2012).

3.1. Use of existing genetic variation

Sexual crosses, bridge crosses, embryo rescue and somatic hybridisation

The most frequently used method of introducing new variation in plant breeding involves the production of viable offspring through the crossing of closely related parental lines selected on the basis of the attributes that the breeder wishes to combine (EFSA, 2012). In this way breeders are able to take advantage of the high degree of structural variation (presence or absence of specific sequences and copy number variation) of individuals within a species (Morgante et al., 2007; Swanson-Wagner et al., 2010). In addition, wild ancestors represent an important source of genetic variation that can be effectively exploited in breeding programmes (Tanksley and McCouch, 1997). The strategy adopted for interspecific crosses is strongly dependent on the biology and evolution of the recipient and donor plant species. Bridge crosses, wide crosses using embryo rescue and somatic hybridisation have been used to overcome barriers to wider hybridisation (Stewart, 1981; Evans, 1983; Mathias et al., 1990; Van Eijk et al., 1991; Lynch et al., 1993; Fedak, 1999). Bridge crosses make it possible to exploit new sources of traits lacking from directly cross-compatible species (Van Eijk et al., 1991; Khrustaleva and Kik, 2000; van der Wiel et al., 2010). When a direct cross between two species is not possible, an intermediate hybrid with a third species, which is compatible with both species, can be used to bridge the crossing barrier. Embryo rescue is used to overcome interspecific incompatibility in plants (Reed, 2005). Embryo rescue deploys *in vitro* culture techniques to assist in the development of plant embryos that might not otherwise survive to become viable plants (Miyajima, 2006). Somatic hybridisation (protoplast fusion) can increase the efficiency of generation of hybrids that can be developed by sexual crossing only with difficulty (Evans, 1983; Glimelius et al., 1991; Liu et al., 2005). When the wild relative's chromosomes are structurally very similar to those of the cultivated species, the chromosomes will pair normally at meiosis during introgression and the desired gene(s) will be incorporated by HR. Spontaneous interspecific chromosome recombinations have been systematically obtained in several crops such as tomato, rice, barley, wheat, maize and cotton and represent valuable pre-breeding material that can be exploited with the help of molecular markers (see Wang and Chee, 2010, for a review).

- Translocation breeding

Translocation breeding is used when the wild relative's chromosome is distantly related to its equivalent in the cultivated species, and uses chromosome engineering strategies to transfer and stably incorporate chromosome segments from a wild ancestor into the cultivated species.

The classic example is the transfer by Sears (1956) of resistance to leaf rust (*Puccinia triticina*) from *Aegilops umbellulata* to *Triticum aestivum* wheat. The key steps were (a) the bridging cross between *T. dicoccoides* (A and B genomes) and *Ae. umbellulata* (C genome), which gave the alloploid AABBCC; (b) the cross between this alloploid and *T. aestivum* (A, B and D genomes), which gave the hybrid AABBCD, in which 14 bivalents and 14 univalents formed during meiosis; (c) two backcrosses to *T. aestivum* using the latter as female parent, and selection of a resistant plant that was AABBDD plus one *Ae. umbellulata* chromosome; (d) selection of a resistant plant in the progeny with an isochromosome that was duplicated in the long arm of the *Ae. umbellulata* chromosome; (e)

treatment of plants carrying the isochromosome with X-rays and use of their pollen on *T. aestivum*; and (f) identification of 40 translocations in 132 resistant plants out of 6 091, of which one was an intercalary translocation that was cytologically undetectable because it formed 21 normal bivalents in meiosis. In the intercalary translocation, a small portion of the *Ae. umbellulata* chromosome had been removed as a result of two DSBs and inserted in a wheat chromosome as a result of one or two DSBs.

From the 1970s to the present day a wide number of wheat varieties carrying a rye translocation have been successfully cultivated worldwide (Rabinovich, 1998). To minimise the presence of undesirable genes that were transferred together with the gene of interest (linkage drag), well-established cytogenetic and molecular methodologies have been successfully applied in wheat breeding programmes (Lukaszewski, 2000; Ceoloni et al., 2005; Gennaro et al., 2007).

While all the techniques described in this chapter are designed to create new combinations of existing genetic variants, some of these processes are also known to induce new mutations. For example, crosses of distantly related species and polyploidisation events have been referred to as genetic shocks inducing genome-wide epigenetic changes and new insertions, deletions or recombinations of transposable elements (Parisod et al., 2010; Yaakov and Kashkush, 2011). Furthermore, the *in vitro* culture steps necessary for embryo rescue and somatic hybridisation techniques have long been recognised as highly mutagenic (see somaclonal variation described below).

3.2. Use of newly created genetic variation

- ***Mutation breeding***

The exploitation of natural or induced genetic diversity is a proven strategy in the improvement of all major food crops, and the use by breeders of mutagenic agents (chemical and physical) to create variation is particularly valuable in those crops with restricted genetic variability or where introgression of genes from wild relatives is difficult and time-consuming. Historically the use of mutagenesis in breeding has involved forward genetic screens and the selection of individual mutants with improved traits and their incorporation into breeding programmes. Since 1960 assessments of the possibilities and limitations of mutation breeding have taken place in many crop species, both seed-bearing and clonally propagated. These have been helped by the establishment in 1964 of internationally co-ordinated research programmes by the joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture. In 2012, the FAO/IAEA mutant variety database listed 3 200 officially released cultivars in over 200 plant species that had been produced by mutation breeding. These included 534 rice lines, 205 wheat lines and 71 maize lines (http://www-infocris.iaea.org or http://mvgs.iaea.org).

Mutagenesis techniques are based on the fact that chemically or physically induced damage to DNA is not always faithfully repaired. For more information on the mechanisms of DSB repair the reader is referred to section 2.1.3. Although the use of chemical and physical mutagens in breeding has clearly been successful, there are two serious limitations to the use of induced physical and chemical mutagenesis. First, most newly introduced mutations are deleterious or strongly linked with other deleterious mutations. Secondly, although not entirely random processes, the techniques of physical and chemical mutagenesis are not specific in the sense that they cannot target predetermined DNA sequences (sites in the genome). An additional difficulty can be the genetic redundancy present in many plant species as a result of gene duplication and polyploidy, such that many mutations have no detectable effect on the plant. Additional details on spontaneous and induced physical and chemical mutagenesis, together with other aspects of modern mutagenesis, are provided below.

a) Spontaneous mutations

Ever since plants were first brought into cultivation, farmers and breeders have selected heritable spontaneous mutations for their phenotypic effects, without understanding their molecular basis. Mutations occur spontaneously in nature but the frequency of such mutations (e.g. not more than 10^{-5}

to 10^{-8} per gene or locus in one plant generation) is too low to rely on alone for accelerated plant breeding. However, many traditionally bred traits in horticultural plants are due to natural mutations (Janick, 2004). For example, natural mutations in single genes of tomato are completely or mostly responsible for its determinate growth habit (Pnueli et al., 1998), resistance to powdery mildew (Bai et al., 2008), and yield heterosis (Krieger et al., 2010). Spontaneous base substitutions, small and large insertions, deletions and rearrangements, replication slippage at repeats (e.g. microsatellites), transposon movement and epigenetic changes have been observed in sexually propagated plants. The recent advances in high-throughput sequencing technologies have allowed more detailed analyses of genomic mutations. Recently in sexually propagated *Arabidopsis thaliana*, the spontaneous mutation rate was estimated to be 7×10^{-9} base substitutions per site per generation, with the majority of base substitutions being G:C→A:T transitions (Ossowski et al., 2010). This is consistent with a cytosine-deamination mechanism and the observation that ultraviolet light mostly causes G:C→A:T transitions at sites where the C is adjacent to another C or a T (Friedburg et al., 2006).

b) Induced mutations

To deliver new plant varieties by mutation breeding, a "trial and error" approach is used to determine the best dose (and dose rate) of the mutagenic agents required to achieve the balance between an overdose that kills too many cells (or seedlings) and a too low dose that results in too few mutants for selection purposes. From a large mutagenised population, extensive selection is subsequently required to identify desirable phenotypes and eliminate the undesirable ones. Additional backcrossing may be required to separate desirable from undesirable mutants before a new cultivar or a new parental line is identified.

Chemical mutagenesis

A large number of chemical mutagens have been discovered with various modes of action (Friedburg et al., 2006). A mutagen may react chemically with DNA (e.g. ethyl methane sulphonate (EMS)) or be converted into a chemical which itself reacts with DNA (e.g. sodium azide) or interferes with DNA replication by mimicking a normal DNA base (5-bromouracil) or base pair (ethidium bromide). The outcome of mutagenesis is affected by the stage of the cell cycle and any repair mechanism that is activated. Thus, the most widely used chemical mutagen, EMS, has more effects than one might expect but can efficiently induce chemical modification of nucleotides, resulting in point mutations, including nonsense (change to stop codon), missense (change to codon for another amino acid) and silent mutations (change to codon for same amino acid which does not generate any modification in phenotype) and splicing mutations.

In *Arabidopsis*, EMS mainly induces C to T changes resulting in C/G to T/A substitutions, as expected. EMS also generates, at low frequency, G/C to C/G or G/C to T/A or A/T to G/C substitution (Krieg, 1963). An estimation of the mutation density indicates that one mutation per 170 kbp is effected by the EMS treatment (Greene et al., 2003), resulting in a considerable number of mutations throughout the genome. Based on codon usage in *Arabidopsis*, the frequency of EMS-induced stop codon and missense mutations has been calculated to be ~5 % and ~65 %, respectively (McCallum et al., 2000). EMS primarily causes single-nucleotide polymorphisms (SNPs), which can alter encoded proteins through premature termination, mis-splicing, and codon changes (Dalmais et al., 2008; Dahmani-Mardas et al., 2010; Piron et al., 2010). In addition to coding sequence changes, polymorphisms in transcription and translation initiation signals were found to alter gene expression (Zhao et al., 2009; Knoll et al., 2011). However, more diverse effects can occur, including major chromosomal aberrations, probably as result of faulty excision repair.

Physical mutagenesis

Mutation can be induced by irradiation with non-ionising (e.g. UV) or ionising radiation (e.g. X-rays, gamma rays (γ-rays), α and β particles, fast and slow neutrons and heavy-ion beams). Physical

mutagenesis was pioneered with X-rays, but γ-rays became more widely used, with 50 % of mutant cultivars carrying genes mutated in this way (http://www.iaea.org). X-rays and γ-rays make direct "hits" on DNA but also have indirect effects from the production of free radicals (reactive oxygen species) in aqueous solution. Heavy-ion beams comprise accelerated ions produced by an ion accelerator such as a cyclotron or synchrotron. They are a novel powerful mutagen because they can induce mutations with high frequency at a relatively low dose at which virtually all plants survive, inducing a broad spectrum of phenotypes without affecting other plant characteristics (Kazama et al., 2011, and references therein).

Gamma-irradiation has been the most widely used type of physical mutagenesis. Recently Morita et al. (2009) assessed 24 gamma-irradiation-induced mutations in six cultivars of japonica rice. Seventeen mutations resulted from irradiation of seed, three from irradiation of flowers and four from irradiation of whole plants. Mutation analysis showed that 15 mutations (62.5 %) were small deletions (1–16 bp; probably the result of one DSB being repaired by NHEJ), four (16.7 %) were large deletions (9.4–129.7 kbp; probably the result of two DSBs occurring simultaneously on the same chromosome, which were rejoined by NHEJ, with loss of segment between the breaks), three (12.5 %) were base substitutions (transversions, indicative of action of reactive oxygen species) and two (8.3 %) were inversions (probably the result of two DSBs occurring simultaneously on the same chromosome, which were rejoined by NHEJ, with inversion of segment between the breaks). Fast neutron radiation has been used for many decades and causes translocations, chromosome losses, and large deletions. Deletions range in size from a few base pairs to several megabases (Li et al., 2001; Men et al., 2002). With regard to heavy-ion mutagenesis, Tanaka et al. (2010) reported 29 carbon ion-induced mutants in two genes of *Arabidopsis*, of which 11 were 1–100 bp deletions, one was a 1 bp insertion, two had base substitutions and 15 had larger rearrangements such as deletions (six in the range 5–230 kbp), inversions, translocations and insertions (breakpoints were indicative of NHEJ). Kazama et al. (2011) irradiated dry *Arabidopsis* seeds with carbon ions and obtained a mutation frequency similar to that achieved with the chemical mutagen EMS, and two and a half times that achieved with X-rays. Analysis of their 22 mutations in five genes revealed 21 null mutations out of 22, four base substitutions (three transversions, one transition), 13 small deletions (< 100 bp), one small insertion and chromosome rearrangements.

Somaclonal variation

The term describes the variation seen in plants that have been produced by plant tissue culture. This variation is particularly common in plants regenerated via a callus phase and has been observed in many plant species. Chromosomal rearrangements are an important source of this variation (see EFSA, 2012). Tissue culture is an important step of many breeding strategies; hence, mutations associated with somaclonal variation will be introduced in many breeding programmes. However, somaclonal variation can also be exploited intentionally as alternative way to create variants and expand the germplasm pool (Larkin and Scowcroft, 1981; Evans, 1989).

Compared with sexually crossed plants, elevated genome-wide mutation rates were shown to occur in regenerant *Arabidopsis* lineages due to somaclonal variation after 1 week of callus phase ($4–24 \times 10^{-7}$ mutations per site) (Jiang et al., 2011). The most common mutations were substitutions, although small indels were also detected. Compared with sexually propagated plants, the ratio of transitions to transversions was very different (0.92 for the regenerant lines vs. 2.41 for sexually propagated plants). These results were confirmed by Miyao et al. (2012). After a 5-month cell culture of rice cells, substitutions, indels and insertions of a retrotransponson were detected. Although the G:C→A:T transition was the most frequent, the ratio of transitions to transversions was only 1.1. The observed mutation rate was $7{,}4 \times 10^{-6}$ per site, which is similar to the estimated mutation rate after *N*-methyl-*N*-nitrosourea (MNU) treatment of rice (Suzuki et al., 2008). This high mutation rate resulted in mutations within 26 exons of rice genes revealing the mutagenic outcomes of tissue culture (Miyao et al., 2012).

3.3. Selection

One main objective of plant breeding is the introduction of genetic variation through the introgression of one or more genes from a donor into the background of an elite variety or through mutagenesis of the existing gene pool and then to recover as much as possible of the elite parent genome. Backcrossing may be used to eliminate defects caused by mutations or by introgression of unwanted genes during the development of new varieties. However, if the genes for undesirable traits are tightly linked to the desired traits, this will lengthen the breeding process as further backcrosses will be required to break the linkage. In some cases it may not be possible to break the linkage by backcrossing. For some plants backcrossing is not a feasible option. This includes plants with a long life cycle (e.g. trees), those which are vegetatively propagated and those which have complex genetic and/or sterility issues (e.g. potato, banana). In the past selection was mainly based on screening of the phenotype, but with the advances of molecular techniques it is now possible to screen mutant populations at the DNA level via, for example, TILLING (targeting induced local lesions in genomes) methods (Sikora et al., 2011).

Selection is a fundamental step in all plant breeding techniques including SDN-3 technique. This will be employed in case of the SDN-3 technique to develop plants that contain only the desired DNA at the target locus.

4. Hazard identification addressing question two of the mandate: Identification of characteristics with the potential to cause adverse effects

When considering hazards related to plants produced using the SDN-3 technique compared with transgenic and conventionally bred plants the major considerations by the EFSA GMO Panel include the source of the DNA and the safety of gene products; alterations to the host genome at the insertion site and elsewhere; the potential presence of non-plant sequences in the insert; and the expression of the trait and its potential wider implications.

4.1. Source of genes and safety of gene products

The SDN-3 technique can be used to integrate DNA to a particular genomic locus. Thus, the resulting plants can be cisgenic, intragenic or transgenic. As indicated in the previous EFSA GMO opinion (EFSA, 2012), hazards arising from the use of a plant-derived gene from a breeders' gene pool by cisgenesis are similar to those from conventional plant breeding, as similar traits result from the modification. When a similar plant-derived gene is used in intragenesis, new combinations of genetic elements may arise that are not found in cisgenic and conventionally bred plants, and these may present novel traits with specific hazards. Hazards can be identified which are specific for transgenic plants as the transgenes and their gene products can be obtained from any source including non-plant sources.

4.2. Alterations to the genome

As DNA is introduced into an exact, predefined location in the plant genome during SDN-3, the hazards arising from random integration are minimised. Alteration to the genome may occur in addition to the targeted integration of the DNA sequences. These alterations can be caused by various processes and mechanisms (see sections 2.1.3 and 3). Undesirable changes occurring in the genome in any breeding approach, whether conventional, transgenic, cisgenic or intragenic breeding, can be removed by backcrossing and/or selection, but exceptions will occur (see section 3.3).

In its opinion on cisgenesis and intragenesis the EFSA GMO Panel addressed different hazards under the following headings (EFSA, 2012):

Alterations to the genome
- *Mechanisms of DNA integration*

- *Sites of DNA integration*
- *Genome disruptions, deletions and rearrangements*
- *Somaclonal variation*
- *Creation of novel open reading frames (ORFs)*

Presence of non-plant sequences in the insert
Modification of gene expression

These issues are also relevant for a comparison of plants developed using the SDN-3 technique with transgenic plants and plants developed using conventional breeding approaches. Since the SDN-3 technique can be used to produce transgenic, cisgenic and intragenic plants and hazards associated with these have already been compared with conventional breeding, the EFSA GMO Panel refers the reader to its previous opinion (EFSA, 2012) for detailed text on each of the topics above. However, with regard to the SDN-3 technique specific points are detailed below.

4.2.1. *Alteration at the insertion site*

The SDN-3 technique aims to introduce DNA into the plant genome. In transgenesis, DNA is integrated into naturally occurring breaks in the plant genome by means of illegitimate recombination (via NHEJ), but there is no preference for specific loci in the genome for the integration process (Alonso et al., 2003). In this respect, during transgenesis, the integration of DNA is essentially random and uses the same mechanisms as naturally occurring recombination processes in plant genomes (EFSA, 2012). By comparison, the aim of the SDN-3 technique is to target DNA insertion to a predefined site in the genome (see section 2.2). Successful targeting of a predefined site by the SDN-3 technique can optimise the genomic environment for gene expression and reduce hazards associated with the disruption of genes and/or regulatory elements in the recipient genome. Furthermore, SDN constructs can be designed to add or exchange specific genes at their native loci (e.g. replacing one disease resistance gene with another from the same gene pool or adding a resistance gene to an existing cluster). Thus, the SDN-3 technique can retain the existing genomic environment surrounding the target locus.

The integration can occur via HR or NHEJ and this can impact on changes induced at the insertion site. In the case of integration by HR the genome alterations at the insertion site will be predetermined by the design of the donor DNA. Therefore, the newly created junctions between the plant DNA and the inserted DNA can be optimised before insertion. Insertion at a target locus by NHEJ can be accompanied by all of the types of genomic changes that have been reported for the NHEJ repair, including DNA deletions, insertions (including “filler” DNA, i.e. short sequences new to the plant), duplications and inversions. Such changes at the insertion site have been observed on many occasions in transgenic plants (EFSA, 2012). Multiple copies of the donor DNA may also be integrated in the plant genome during NHEJ.

In summary, hazards that might arise from insertion at a random locus can be reduced with the SDN-3 technique. When DNA is inserted using of the SDN-3 technique and HR the genome alterations at the insertion site will be predetermined by the design of the donor DNA, whereas after targeted NHEJ genomic changes due to DNA repair will be similar to those observed in currently developed transgenic plants.

4.2.2. *Alteration elsewhere in the genome*

The development of SDN-3 plants may include a tissue culture phase (see section 2.2.1). As with other breeding techniques, when tissue culture is used, somaclonal variation (see section 3.1) may induce changes in the genome. This was already dealt with in the EFSA’s opinion on cisgenesis and intragenesis (2012) and is therefore not discussed in detail here.

In general, the use of SDN-3 technique will change the genomic sequence of a target plant in a very specific way. However, changes elsewhere in the genome can occur as a result of off-target

modification. Off-target activity of the SDN depends on its specificity and the presence and accessibility of sequences similar to the SDN recognition site in the genome. For example, there may be sufficient similarity at a non-target site in the genome for SDN to cleave the DNA but insufficient sequence similarity to the donor DNA for HR to occur. In these cases repair will be via NHEJ, which can result, as in conventional breeding and transgenesis, in indels, inversions and translocations (see section 2.1.3). Moreover, integration of the donor DNA at a non-target locus is possible, but as previously stated, end products containing these sequences at a non-target locus will not fall under the SDN-3 definition.

Several methods have been developed to reduce off-target modifications (see section 2.1.1). These should significantly reduce the frequency of such events compared with conventional breeding. In addition, the SDN strategy can be tested *in vitro* and optimised for off-target activity. In the context of the SDN-3 technique, a comparison with both chemical and physical mutation breeding is relevant because of potential off-target effects and the need for optimisation of the system before use (discussed in section 3.2). Physical mutagenesis, ionising or non-ionising, induces DNA damage relatively randomly and causes many types of mutations including base substitutions, deletions and chromosomal alterations (Cecchini et al., 1998; Morita et al., 2009). Gamma-irradiation has been the most widely used type of physical mutagenesis and the majority of changes introduced are small deletions although large deletions, base substitutions and inversions also occur (Morita et al., 2009). Fast neutron radiation has been used for many decades and causes translocations, chromosome losses, and large deletions. In contrast the use of chemical agents such as EMS mostly results in point mutations including nonsense, missense and silent mutations. Both physical and chemical mutagenesis results in a considerable number of mutations throughout the genome with the nature of the changes influenced by the method used. SDNs would induce fewer unintended changes than most conventional mutagenesis techniques, and where they do occur the changes would be of the same types as those produced by conventional breeding techniques.

In summary, changes elsewhere in the plant genome can occur as a result of off-target activity of the SDN and because of the tissue culture step that may be employed during the SDN-3 process. After off-target activity by the SDN, the DSB will be repaired by the plant, which can result in small deletions/insertions/substitutions or larger rearrangements. Such changes also occur naturally after repair of spontaneous DSBs. The frequency of mutations will probably be higher after mutation breeding (as defined in section 3.2) than with the SDN-3 technique.

4.3. Conclusion

Hazards that might result from various plant breeding techniques are related to the source of genes used, the genes and traits deployed and changes to the structure, organisation and sequence of the recipient genome. The primary drivers are the genetic alterations that various breeding processes introduce into the plants, as all other changes that take place are direct or indirect consequences of these changes. Hazards regarding these alterations may arise both in conventional breeding and in transgenesis.

The ZFN-3 technique, and SDN-3 in general, is used for targeted insertion of DNA. With respect to the genes introduced, the SDN-3 technique does not differ from the other genetic modification techniques currently used, and can be used to introduce transgenes, intragenes or cisgenes. The hazards related to the source of genes have been described by EFSA (EFSA, 2012).

The SDN-3 technique makes use of the same transformation techniques as transgenesis, although both transient and stable expression of the SDN can be used to introduce the site-specific DSB. In the case of stable integration of the SDN genes, they can subsequently be removed by segregation to obtain plants containing only the integrated gene. The main difference between the SDN-3 technique and transgenesis is that the insertion of DNA is targeted to a predefined region of the genome. Therefore, the SDN-3 technique can optimise the genomic environment for gene expression and minimise hazards associated with the disruption of genes and/or regulatory elements in the recipient genome.

The SDN-3 technique may introduce modifications at other places in the genome due to off-target activity of the SDN enzymes. This will depend on the specificity of the SDN and the presence of sequences similar to the SDN recognition site in the recipient genome. Several methods have been developed to reduce off-target modifications which significantly reduce the frequency of such events. In addition, the SDN-3 technique can be tested *in vitro* and optimised for off-target activity. If the SDN-3 technique used to produce the plants includes a tissue culture step, unintended changes in the genome can occur as a result of somaclonal variation (as in conventional breeding and transgenesis when tissue culture is used). However, the SDN-3 technique would induce fewer off-target changes than most mutagenesis techniques and, where they do occur, the changes would be the same types as those produced by conventional breeding techniques.

5. Applicability of the current guidance with respect to the risk assessment of SDN-3 technique

The EFSA GMO Panel has detailed in its guidance documents how to comply with the requirements set out in Directive 2001/18/EC. In order to assess the adequacy of the current EFSA guidance documents for the risk assessment of plants developed using the SDN-3 technique, the EFSA GMO Panel has focused on the *Guidance for risk assessment of food and feed from genetically modified plants* (EFSA, 2011) and the *Guidance on the environmental risk assessment of genetically modified plants* (EFSA, 2010).

In its opinion on cisgenesis and intragenesis (EFSA, 2012), the EFSA GMO Panel has outlined the concepts of "*history of safe use"* for consumption as food and the concept of familiarity as important components of the risk assessment approach. It has also described the main components of the guidance documents used for risk assessment.

The EFSA GMO Panel considers that the general approach and all elements, described in the *Guidance for risk assessment of food and feed from genetically modified plants* (EFSA, 2011) and the *Guidance on the environmental risk assessment of genetically modified plants* (EFSA, 2010), are applicable for the evaluation of food and feed products derived from plants developed using SDN-3 technique and for performing an environmental risk assessment and do not need to be developed further. However, and as already discussed (EFSA, 2012), it can be envisaged that on a case-by-case basis (e.g. where the SDN-3 technique is used for cisgenesis) lesser amounts of event-specific data are needed for the risk assessment. Furthermore, for plants produced using a successful SDN-3 strategy there will be cases where the potential for unintended effects e.g. positional and off-target effects, is significantly reduced compared with transgenesis, and with conventional breeding. There is therefore a need for flexibility in the data requirements for risk assessments.

CONCLUSIONS

The EFSA GMO Panel compared the hazards associated with plants produced by the SDN-3 technique with those associated with plants obtained by conventional plant breeding techniques and by currently used transgenesis.

The main difference between the SDN-3 technique and transgenesis is that the insertion of DNA is targeted to a predefined region of the genome. Therefore, the SDN-3 technique can optimise the genomic environment for gene expression and minimise hazards associated with the disruption of genes and/or regulatory elements in the recipient genome.

The SDN-3 technique can induce off-target changes but these would be fewer than those occurring with most mutagenesis techniques. Where they do occur, the changes would be the same types as those produced by conventional breeding techniques.

With respect to the genes introduced, the SDN-3 technique does not differ from the other genetic modification techniques currently used, and can be used to introduce transgenes, intragenes or cisgenes.

The EFSA GMO Panel considers that the Guidance for risk assessment of food and feed from genetically modified plants (EFSA, 2011) and the Guidance on the environmental risk assessment of genetically modified plants (EFSA, 2010) are applicable for the evaluation of food and feed products derived from plants developed using the SDN-3 technique and for performing an environmental risk assessment. However, on a case-by-case basis lesser amounts of event-specific data may be needed for the risk assessment of plants developed using the SDN-3 technique. There is therefore a need for flexibility in the data requirements for risk assessments.

GLOSSARY

Definitions as used in this document:

Agrobacterium tumefaciens	A naturally occurring pathogenic bacterium (also known as *Rhizobium radiobacter* (Young et al., 2001)) of plants that can transfer a part of its DNA into plant cells.
Artificial target locus	A locus that has been introduced via transformation and can then be targeted by site-directed nucleases.
Backcross	A cross between a hybrid and one of its parents. Subsequent backcrosses of offspring to same (recurrent) parent produce offspring of increasing similarity to that parent.
Cisgenesis	The genetic modification of a recipient organism with a gene from a crossable – sexually compatible – organism (same species or closely related species) (EFSA, 2012).
Donor DNA	DNA that is introduced into plant in order to serve as a template during genome editing or gene targeting.
Embryo rescue	A tissue culture technique used to enable an excised immature embryo, usually from an interspecific cross, to continue growth and development following 'endosperm collapse'.
Endonuclease	An enzyme that cleaves the phosphodiester bonds within nucleic acid molecules.
Epigenetic	Refers to alterations of gene activity without altering the nucleotide sequence or genotype of an organism.
Gene pool	The totality of the genes and alleles of a defined population of organisms at a given time (EFSA, 2012).
Genetically modified	Refers to an organism whose genotype has been altered in a way that does not naturally occur (see Directive 2001/18/EC).
Germplasm	Collection of genetic stocks (genotypes) of an organism.
Hazard	Any source that has the potential to cause an adverse effect on human health, animal health or the environment.
Homologous recombination	Recombination between highly similar DNA sequences (Lawrence, 1995).
Intercalary translocation	Insertion of a small portion of a chromosome into another chromosome.
Intragenesis	Intragenesis is a genetic modification of a recipient organism that leads to a combination of different gene fragments from donor organism(s) of the same or a sexually compatible species as the recipient (EFSA, 2012).
Introgression	The introduction of new alleles or genes into a population from an exotic source, usually another species, by recurrent backcrossing.
Isochromosome	A chromosome with two identical arms.
Linkage drag	Co-transfer of DNA sequences that are linked to the gene of interest.
Locus	Physical: the specific position of a gene or a DNA sequence on a chromosome. Genetic: the position of a gene on the genetic map.

Mutations	Any detectable and heritable change in the genetic material not caused by segregation or genetic recombination, that is transmitted to daughter cells and even to succeeding generations, giving rise to mutant cells or mutant individuals provided it does not act as a dominant lethal factor (Rieger et al., 1968).
Nickase enzyme (ZF-nickase)	Enzyme that cuts one strand of a double-stranded DNA to produce DNA molecules that are "nicked", rather than cleaved.
Off-target activity of SDN	Activity of the SDN at sites different from the target site in the genome due to limited specificity of the SDN.
Phenotype	The observable properties (structural and functional) of an organism, produced by the interaction between the organism's genetic potential (genotype) and the environment in which it finds itself (Rieger et al., 1968).
Polyploid	Somatic cells and tissues, as well as individuals with more than two chromosome sets (genomes) (e.g. triploid (3×) tetraploid (4×) pentaploid (5×) or hexaploid (6×)). Polyploidy is described as autopolyploidy if the chromosome sets are derived from the same or similar genomes and allopolyploidy if they are the combination of genomes from different species.
Promoter	A segment of DNA to which RNA polymerase attaches, allowing the initiation of the transcription of a gene. It usually lies upstream of (5' to) a gene (adjusted from Glick and Pasternak (2003)).
Protein domain	A discrete portion of a protein with its own function. The combination of domains in a single protein determines its overall function.
Protoplast	The protoplasm of a single cell, obtained by the enzymatic digestion of the cell wall (adapted from Mauseth (1991)).
Recombination	The creation, by a process of intermolecular exchange, of chromosomes combining genetic information from different sources. Site specific, homologous, transpositional and non-homologous illegitimate) types of recombination are known.
Segregation	The separation of allele pairs from one another and their distribution to different cells, usually at meiosis and sometimes at mitosis (Rieger et al., 1968).
T-DNA	DNA encoded on a plasmid of *Agrobacterium* that is transferred to the plant cell.
Transgenesis	Currently used techniques for the introduction in a non-targeted manner of genetic information into cells that leads to the transmission of the input gene (transgene) to successive generations.
Transposon	A DNA element capable of moving (transposing) to a new genomic location in the same cell.

List of abbreviations

bp	base pair
CA	Competent Authority of a Member State
DNA	deoxribonucleic acid
DSB	double-strand break
EC	European Commission
EMS	ethyl methane sulphonate
GMO	genetically modified organisms
HR	homologous recombination
indel	insertions/deletions
kbp	kilobase pairs
MN	meganuclease
NHEJ	non-homologous end-joining
nt	nucleotide
NTWG	New Techniques Working Group of the Competent Authorities under Directive 2001/18/EC
PM	point mutation
RVD	repeat-variable di-residue
SDN	site-directed nuclease
SDN-3	site-directed nuclease 3 technique
SSB	single-strand break
TAL	transcription activator-like
TALEN	transcription activator-like effector nuclease
ZF	zinc finger
ZFN	zinc finger nuclease

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