POSITION PAPER

Site Directed Nucleases (SDN) for targeted genome modification

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Note: This document is intended to serve as a reference for characterizing various technical outcomes of gene editing as either "SDN-1", "SDN-2" or "SDN-3", categorizing them by the mechanism of the DNA repair process and the respective outcome. "SDN-1", "SDN-2" and "SDN-3" classification is not fully applicable to illustrate an appropriate regulatory policy approach associated with each of these categories, i.e., it is not fully transposable to serve as a basis for defining a regulatory "cut-off" which category results in a GMO (regulated) organism, particularly concerning the variety of SDN-3 outcomes.

Crop improvement through breeding crosses uses genetic variation to create new combinations of characteristics. The development of a new plant variety through conventional breeding requires consecutive rounds of crossing and selection that integrates existing and new genetic variation to yield a plant with desired combination of characteristics. The use of mutagenesis can further expand the range of variation in the breeding pool by increasing the frequency of nucleotide changes in the plant's genome. One significant limitation to crop improvement through conventional breeding methods is the amount of time required to identify, cross, and select for a desired characteristic.

The advancement of targeted genome editing methods complements and further expands existing breeding tools by enabling more efficient introduction of desired sequence changes (genetic variation) in specific genes. Many targeted genome editing methods are based on the ability to induce a DNA double strand break at a selected location in the genome. Repair of this double strand break by the plant's own cellular repair mechanisms results in the introduction of the desired changes, or targeted genome editing.

Various tools can be used to achieve targeted DNA double strand break, and those used up to now include homing endonucleases (also called meganucleases), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)-associated proteins (e.g., CRISPR/Cas9 and CRISPR/Cpf1) [Gaj et al., 2013, Podevin et al., 2013; Lee et al., 2016; Weeks et al., 2016; Endo et al., 2016]. Collectively, these tools are often described under the term "site directed nucleases" (SDNs), pointing out the general principle of the technology to use a DNA cutting enzyme (nuclease) for the generation of the targeted (i.e., site directed) double strand break facilitating targeted genome modification.

The outcomes of SDN applications are often categorized as SDN-1, SDN-2 and SDN-3 using a combination of two criteria: 1) the nature of the underlying cellular repair process of the DNA double strand break and 2) the type of the introduced genetic change.

1) Cells can repair DNA breaks via one of two cellular repair pathways. These same pathways also mediate the repair of DNA double strand breaks generated with the genome editing tools described above. For SDN-1 the repair process follows the non-homologous end joining (NHEJ) pathway. For SDN-2 and SDN-3 the repair process follows the homologous recombination (HR) pathway which is dependent on the presence of a homologous sequence (repair template) to guide the repair process [Podevin et al., 2013; Pacher and Puchta, 2017].

2) Because the range and type of possible changes introduced via the NHEJ or HR repair pathways differ, a second criterion, the type of sequence change, is used to further distinguish the outcomes.

It is important to consider that while the classification provides a useful structure for technical and regulatory discussions, it does not allow full discrimination between the categories as there are some overlaps in terms of type of possible sequence changes. These are discussed in more detail below and summarized in Table 1.

SDN-1: is the use of site directed nuclease to generate a targeted DNA double stranded breaks at specified genetic sequence corresponding to a gene coding or a regulatory region and the subsequent DNA repair not involving a repair template. Such repair can result in small nucleotide deletions, additions or substitutions at the break site. Larger deletions (e.g., deletion of the entire gene) can be achieved when two DSBs are induced on either side of a targeted DNA sequence. Screening of SDN-1- mutagenized plants allows for selection of a plant with sequence changes leading to a desired characteristic. SDN-1 mediated mutations are analogous to those generated by spontaneous or induced mutagenesis [Schnell et al., 2015; Pacher and Puchta, 2017]. The most common use of the SDN1 tool is to generate deletions in order to knock-out gene function; it can be also used to alter gene expression by targeted deletions in the promoter region [e.g., Haun et al., 2014; Jia et al., 2016; Sanchez-Leon et al., 2017, Li et al., 2012; Nonaka et al., 2017].

Compared to conventional breeding techniques, SDN-1 provides for more predictable outcomes by specifying the sequences to be mutagenized, thus reducing the need to screen large populations of plants to select those with desired changes. Additionally, SDN-1 application has proven to be efficient in generating mutations in polypoid crops and crops with limited native genetic diversity [Weeks, 2017]. These advantages allow efficiency gains and save time in the breeding process.

SDN-2: is the use of site directed nuclease to generate a targeted DNA double strand break followed by the double strand break repair using a repair template which represents the DNA sequence homologous to the targeted DSB DNA sequence. In one application of SDN-2 technology, the repair template may define a single or more nucleotide changes that would lead to the change of corresponding amino acid(s) [Svitashev et al., 2015; Li et al., 2016]. SDN-2 can be also used for optimizing the target gene's regulatory region or replacing the entire allele with an alternative allele of the same gene from another variety [Podevin et al., 2013, Custers, 2017, DuPont Pioneer, 2017].

Compared to conventional breeding techniques, SDN-2-provides for more predictable outcomes thus reducing the need to screen large populations of plants to select those with desired changes. Additionally, SDN-2 application may allow plant breeders to optimize desired alleles directly in elite breeding lines which would lead to a reduction in the number of breeding crosses needed to introduce the desired change [Jones, 2015].

SDN-3: is the use of site directed nuclease to generate a targeted DNA double strand break followed by repair of the double strand break with a DNA template that enables targeted insertion (addition) of new DNA sequences at a selected genome location. Similar to SDN-2, SDN-3 leads to a predefined change in the genome as the position of the DNA double strand break and the sequence change upon its repair are predetermined. However, in contrast to SDN-2, the sequence added via the repair template in SDN-3 includes a new sequence at that particular genomic location. The new insertion can include sequences derived from within or outside the gene pool of the species, such as other plants or bacteria.

An important application of SDN-3 is the ability to efficiently introduce native genes or regulatory sequences derived from within the species' genetic pool. For example, targeted addition of a copy of a native gene results in higher gene copy number that may allow increase in protein production; targeted addition of a promoter from the same species' genetic pool could result in modulation of gene expression [Shi et al., 2017]. These outcomes are similar to those occurring in nature, in particular due to activity of transposable elements [Pacher and Puchta, 2017; Glenn et al. 2017; Lal et al., 2009] or phenomena of the gene copy number variation (CNV) and gene presence/absence variation (PAV) [Springer et al., 2009; Da Silva et al., 2013].

SDN-3 can also be used to insert transgenes or sequences outside of the species' genetic pool [Shukla et al., 2009; Svitashev et al., 2015]. SDN-3 mediated transgene insertions could be a method of choice for stacking several transgenic traits together so that they are inherited as a single genetic locus [Ainley et al., 2013; Kumar et al., 2015; Chilcoat et al., 2017]. SDN-3 mediated insertions could help reduce the time needed for trait introgression and enables efficient incorporation of traits into elite lines for the development of new varieties.

SDN application	Targeting to specific genome location?	Use of repair template? Origin of the repair template	Type of targeted sequence change(s)
SDN-1	YES defined by nuclease specificity	NO	**Mutation(s)** (spontaneous mutations: deletions, replacements, additions of sequence)
SDN-2	YES defined by nuclease specificity	YES species own gene pool only	**Edit(s)** (predefined mutations, sequence optimization, allele replacement)
SDN-3	YES defined by nuclease specificity	YES any source, including species own gene pool	**Insertion(s)** (addition of sequence at the target genomic location)

Modifying native gene sequence and function, and introgression of genetic variability using the species' genetic pool are similarly possible through conventional breeding methods. Targeted mutagenesis (SDN-1), gene editing (SDN-2) or certain applications of targeted insertion (SDN-3) produce the same types of outcomes as possible with other breeding methods.

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