Rationale for the equivalence criteria in Annex I to the proposal for a Regulation on plants obtained by certain new genomic techniques

Technical paper by the Commission services

This technical paper provides detailed explanation on the rationale for the criteria in Annex I of the proposal for a regulation on plants obtained by certain new genomic techniques (NGTs) and their food and feed (in the following "the NGT proposal")[1], and presents information on the relevant scientific literature.

The criteria were developed to define type and number of mutations introduced by targeted mutagenesis and cisgenesis that could also be obtained by conventional breeding methods or could occur spontaneously. They were developed on the basis of a literature analysis of 90 scientific, peer-reviewed original studies and reviews (see Annex) on plants obtained by conventional breeding methods[2] and on genetic variations in plants. The objective of the analysis was to explore:

- Which type of mutations occur due to natural mutation or application of conventional breeding methods.
- What size ranges these mutations span.
- How many of these mutations do typically occur in a single plant.

Also relevant considerations in scientific opinions issued by the European Food Safety Authority (EFSA)[3] and scientific work of the Joint Research Centre (JRC)[4] were taken into account in defining the criteria.

Similar genetic modifications obtained by different techniques are not expected to present different risks. Therefore, this analysis was not meant to assess the effects of genetic variations or genetic modifications introduced by conventional breeding methods.

1. Nature of the criteria

The criteria are based on the modifications resulting from the technique(s), i.e., on molecular characteristics. Furthermore, if certain type and number of mutations can be introduced by both conventional breeding techniques and NGTs, also the type of traits associated to these mutations

[1] COM(2023) 411 final

[2] For a description of conventional breeding techniques in plants, see e.g.:

- EFSA Panel on Genetically Modified Organisms (GMO). (2012). Scientific opinion addressing the safety assessment of plants developed using zinc finger nuclease 3 and other site-directed nucleases with similar function. EFSA Journal, 10(10), 2943. https://doi.org/10.2903/j.efsa.2012.2943
- European Commission, Directorate-General for Research and Innovation, New techniques in agricultural biotechnology, Publications Office, 2017, https://data.europa.eu/doi/10.2777/574498.

[3] EFSA Panel on Genetically Modified Organisms, 2012. Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis. EFSA Journal 2012;10(2):2561.
EFSA Panel on Genetically Modified Organisms, 'Applicability of the EFSA Opinion on SDNs type 3 for the safety assessment of plants developed using SDNs type 1 and 2 and oligonucleotide-directed mutagenesis', EFSA Journal 2020;18(11):6299. https://doi.org/10.2903/j.efsa.2020.6299
EFSA Panel on Genetically Modified Organisms, 2022. Updated scientific opinion on plants developed through cisgenesis and intragenesis. EFSA Journal 20(10):7621, 33 pp. https://doi.org/10.2903/j.efsa.2022.7621.
EFSA Panel on Genetically Modified Organisms, 2022. Statement on criteria for risk assessment of plants produced by targeted mutagenesis, cisgenesis and intragenesis. EFSA Journal 20(10):7618, 12 pp. https://doi.org/10.2903/j.efsa.2022.7618

[4] Broothaerts, W., Jacchia, S., Angers, A., Petrillo, M., Querci, M., Savini, C., Van den Eede, G. and Emons, H., New Genomic Techniques: State-of-the-Art Review, EUR 30430 EN, Publications Office of the European Union, Luxembourg, 2021, ISBN 978-92-76-24696-1, doi:10.2760/710056, JRC121847

would not be different between the techniques. Therefore, for the purpose of assessing equivalence, the analysis of type and number of mutations is considered sufficient.

2. Justification of the type of genetic modifications included in the criteria

The literature analysis as described in the Annex showed that targeted mutagenesis and cisgenesis techniques can lead to genetic modifications that are similar to mutations occurring spontaneously in nature or as a result of conventional breeding techniques, including random mutagenesis techniques using chemicals or various types of irradiation. These mutations include substitutions, insertions (including duplications, translocations and inversions) and deletions of nucleotides in the DNA. Furthermore, insertions of cisgenes[5] or parts of cisgenes are also possible through crossing or conventional breeding. These types of mutations are observed also in combination.

In view of these results and EFSA's conclusion that targeted mutagenesis and cisgenesis (with the exclusion of intragenesis) do not *per se* generate specific hazards different to those from conventional breeding methods, targeted substitutions, insertions and deletions (criteria 1 and 2 of Annex I of the NGT proposal), targeted insertions and substitutions of cisgenes (criterion 3) as well as targeted inversions (criterion 4) were included among the criteria of equivalence. Criterion 5 was included to consider possible outcomes (DNA sequences) that might be shown to occur in a species from the breeders' gene pool[6] but that might not be covered by the previous criteria. This criterion provides a derogation only from criterion 3 and from the condition that the genetic modification does not interrupt an endogenous gene.

Based on EFSA's conclusion that intragenic plants[7] may entail additional hazards compared to conventionally bred plants, intragenesis was excluded from the criteria by setting, under criterion 3, the two conditions of (i) no interruption of an endogenous gene[8] and (ii) insertion of (criterion 3a) or substitution with (criterion 3b) a contiguous DNA sequence. The random insertion of a cisgene was also excluded to take into account EFSA's opinion that an interruption of an endogenous gene by a cisgene may give rise to additional hazards that would require assessment. As regards criteria 1 and 2, they also only cover targeted modifications, since random mutagenesis is already exempted from the application of the GMO legislation and is not in the scope of the NGT proposal.

3. Justification of the size limits of individual genetic modifications included in the criteria

On the basis of the literature research, it appears that substitutions occurring after application of conventional methods typically affect one or few adjacent nucleotides. Insertions resulting from these methods can span up to several million nucleotides in cases of structural rearrangements like duplications or translocations of sequences already present elsewhere in the genome. Insertions of more random sequences are typically of a length of less than ten nucleotides but have been observed to extend to approximately fifty nucleotides. Furthermore, although smaller insertions so far have been reported to occur more frequently than larger rearrangements, the improvement of detection methods (i.e. long-read sequencing) has started to unveil higher rates of large insertions than previously estimated. Deletions of less than fifty nucleotides seem to be the most common, but deletions affecting large regions of plant genomes spanning up to several hundred or thousand nucleotides have also been observed. Finally, as with other structural rearrangements, inversions of several million nucleotides have been reported to occur after use of conventional methods.

[5] A gene originating from the same or a crossable species.
[6] For the definition of breeders' gene pool see COM(2023) 411 final, Article 3(6).
[7] Plants containing a rearranged copy of genetic material originating from the same or a crossable species.
[8] An endogenous gene is a gene present in the target organism.

Considering these findings in the literature, no thresholds for the lengths of admissible deletions and inversions were set in criterion 2 and 4, respectively.

In contrast, a threshold of twenty nucleotides in criterion 1 for substitutions and insertions was set since it fits with the sizes observed in the scientific analysis. The described very large insertions as part of structural rearrangements should be considered insertions of a cisgene, which are covered by criterion 3. Insertions of random sequences were reported to be much smaller. Furthermore, when considering genome diversity, the JRC calculated that the theoretical probability that a random sequence is unique in the genome of various crops boils down to a consistent relatively narrow size range between 19 and 21 bases[4]. This means that a modified sequence smaller than this size may already occur elsewhere in the genome and may therefore be already part of the natural genetic diversity.

As regards the threshold for the length of substitutions, the same threshold as for insertions of random sequences has been applied since substitutions can be considered as a combination of deletion and insertion. Any deviation between the threshold applicable to insertions and the one applicable to substitutions would thus have created an inconsistency.

4. Justification of the numerical limit of individual genetic modifications per plant included in the criteria

As regards the total number of modifications in an individual plant introduced by conventional breeding techniques, the literature illustrated variability depending on various factors, in particular the organism and the method used. In general, higher doses of chemicals or radiation and longer exposure times increased the number of genetic modifications in individual plants. Additionally, polyploid plants[9] tended to exhibit greater numbers of genetic modifications compared to monoploid plants. Typically, from the literature analysis, the total number of genetic modifications in individual viable plants ranged from thirty to one hundred. The mutation frequency after using random mutagenesis was higher compared to natural mutation rates. It remained nevertheless below the total number of accumulated single nucleotide polymorphisms[10] naturally occurring between different cultivars or the number of genetic mutations resulting from conventional methods using tissue culture, clonal propagation or protoplast regeneration.

Through conventional breeding, new or improved cultivars are obtained by stacking genes or making new genomic combinations. However, these techniques are more successful for some crops and genes than for others. While in general stacking of multiple desirable modifications is possible with conventional methods, there are several examples where conventional breeding is not effective in this respect, due to a number of factors. The probability to achieve specific, potentially more extensive, combinations of modifications as a result of the application of conventional methods may be low[11]. Based on this, a limit to the total number of individual modifications per plant was set to twenty in Annex I of the NGT proposal. By this threshold, a demarcation is drawn so to exclude from category 1 of the proposal NGT plants with complex modifications unlikely to be obtainable by conventional breeding methods.

The increasing precision of certain NGTs compared to conventional breeding approaches is well recognised in the scientific community[12]. However, to consider in the verification of equivalence all

[9] Plants containing more than two homologous chromosomes.
[10] Substitutions at a single position in the genome.
[11] For example, traditional mutagenesis and plant breeding have not been effective in obtaining low gluten wheat varieties for patients with coeliac disease due to the significant number of specific mutations required to obtain such a trait. Targeted mutagenesis has been instead used to precisely and efficiently reduce the amount of gluten in wheat seed kernels (Sanchez-Leon et al., 2018).
[12] SWD(2023) 412 final, section 1.1 and Annex 6.

possible modifications introduced by the use of the new techniques, the limit of 20 to the total number of individual modifications per plant is set to cover not only the on-target genetic modifications but also possible off-target modifications occurring in DNA sequences sharing sequence similarity with the targeted site that can be predicted by bioinformatic tools.

Annex

Analysis of the scientific literature on mutations occurring naturally or obtained by conventional breeding techniques

1. Introduction

The Commission's services analysed scientific, peer-reviewed literature regarding the type, size and occurrence of mutations, as well as the number of mutated genes, which mainly focuses on random mutagenesis techniques such as irradiation and the application of EMS. The mutations induced by random mutagenesis techniques (e.g. using ethyl methanesulfonate (EMS), gamma ray irradiation, fast-neutron (FN) irradiation) include **nucleotide substitutions, insertions and deletions** of various sizes. Mutations introduced by these techniques are comparable to mutations derived from certain NGTs in which breaks are induced in the DNA and edits result from imperfections in the natural DNA repair mechanism of plants (EFSA GMO Panel, 2012; Pacher and Puchta, 2017; Holme, Gregersen & Brinch-Pedersen, 2019; EFSA GMO Panel, 2020). According to the consulted literature, random mutagenesis techniques lead to a lower number of mutations compared to e.g. *in vitro* breeding techniques such as tissue culture and clonal propagation (Zhang *et al.,* 2014; Adamek *et al.*, 2022). Also, the natural variation found in existing cultivars, generated over time by natural and breeding processes, is much larger than the number of mutations induced by random mutagenesis (Anderson *et al*., 2016).

The types of mutations described below have been observed in the literature analysis. Combination of these types of mutations were also observed (Belfield *et al*., 2012; Hase *et al*., 2023; Li *et al*., 2016b; Weng *et al*., 2019).

2. Type and size of mutations caused by random mutagenesis techniques

2.1. Substitutions

Single base substitutions (SBSs), i.e. the replacement of a single nucleotide or a few adjacent nucleotides in the DNA, were the most common group of edits when using FN irradiation in rice (52.6% of observed mutations) (Li *et al*., 2016a). The majority of mutations in carbon ion-irradiated *Arabidopsis thaliana* also constituted SBSs (38%-43% in dry seed and 59%-62% in seedlings) (Hase *et al*., 2018). SBSs were also four times more frequent compared to short insertions or deletions (indels) in six gamma-irradiated rice lines, where they were randomly distributed over the genome (Li *et al*., 2016b). In addition, the application of EMS for random mutagenesis in *Arabidopsis*, soybean and rice led predominantly to SBSs (e.g. more than 99% of mutations are G/C to A/T transitions in *Arabidopsis*) (Greene *et al.,* 2003; Cooper *et al*., 2008; Henry *et al*., 2014). These results indicate that SBSs are commonly observed as a result of random mutagenesis.

2.2. Deletions

Although SBSs were the most abundant mutations overall in FN-irradiated rice, the largest fraction of mutated genes (71.5%) harboured deletions. Of these, small deletions were the most abundant, with 26.3% comprising a single base pair (bp). Nevertheless, several deletions exceeding a size of 1 kilobase pair (kbp) were also observed and the largest deletions spanned several hundred kbp (Li *et al*., 2016a). The most frequent mutation type in 24 rice plants with a mutant phenotype after gamma ray irradiation were small deletions of up to 16 bp (62.5%), but larger deletions ranging in size between 9 and 130 kbp were also noted (16.9%) (Morita *et al*., 2009). Large deletions were also observed in 264 FN-irradiated soybean lines, where on average two to three homozygous deletions of more than 500 bp were detected per line (Bolon *et al*., 2014). In five FN-irradiated common bean plants, large deletions were found to range in size from 40 bp to 43 kbp (O´Rourke *et al*., 2013). The majority of the deletions in carbon ion-irradiated dry seed and seedlings of *Arabidopsis* were below

50 bp in size (95% in dry seed and 91.5% in seedlings), but larger deletions of more than 1 kbp were also observed (Hase *et al*., 2018). A recent study of the same group comparing carbon ion and gamma ray irradiation of dry seed and seedlings of *Arabidopsis* produced similar results with mainly deletions of less than 10 bp but also several instances of large deletions of more than 100 bp particularly when using carbon ion irradiation. The largest deletion found had a size of 380 kbp (Hase, Satoh & Kitamura, 2023). Likewise, in FN-irradiated *Arabidopsis* most of the deletions (97%) were smaller than 56 bp in length, with single base deletions being the most frequently observed (36%). However, a larger deletion of 7.2 kbp was also identified (Belfield *et al*., 2012). In a similar study, Li *et al*. (2001) found that in FN-irradiated *Arabidopsis* lines most of the deletions were of a size of up to 4 kbp (58.3%). However, deletions as large as 12 kbp were also found (Li *et al*., 2001). Large deletions of up to 35 kbp resulting from FN irradiation of *Arabidopsis* were also reported in other studies (summarised in Li & Zhang, 2002).

In conclusion, although small deletions seem to be more abundant when using random mutagenesis techniques, large deletions of several kbp also frequently occur.

2.3. Insertions

Although insertions are less frequently reported in comparison to deletions, they do occur. For instance, in FN-irradiated rice most insertions identified were 1 bp long (69%). Insertions of 2-6 bp were also observed (27%), while insertions of more than 10 bp were rare (Li *et al.,* 2016a). In carbon ion-irradiated *Arabidopsis*, single base insertions were most frequent (18 out of 35 insertion events), seven events were insertions of 2 bp and the remainder ranged between 3 and 47 bp (Hase *et al*., 2018). However, larger insertions of more than 50 bp also occur, which are classified as several subtypes of Structural Variations (SVs), for instance translocations, inversions and duplications (Saxena, Edwards & Varshney, 2014; Huang & Rieseberg, 2020; Zanini *et al.,* 2021). For instance, in 264 FN-irradiated soybean plants on average one segmental duplication with an average size of more than 2 megabase pairs (Mbp) was identified per mutant line (Bolon *et al*., 2014). Similarly, two studies using carbon ion or gamma ray irradiation on *Arabidopsis* revealed several inversions and translocations of various sizes (Hase *et al.,* 2018; Hase, Satoh & Kitamura, 2023).

Recent reports suggest that the prevalence of short-read sequencing techniques as the standard detection method for genetic mutations may have led to an underrepresentation of the number and size of larger SVs in the genomes of plants subjected to random mutagenesis techniques (Sedlazeck *et al*., 2018; De Coster & Van Broeckhoven, 2019; Ho, Urban & Mills, 2020; Zanini *et al.,* 2021; Lemay *et al.*, 2022; Zhang *et al.*, 2022). Also, it was suggested that the combination of multiple algorithms for data analysis may improve the detection rate of SVs (Hase, Satoh & Kitamura, 2023). Indeed, several recent studies reported a much higher than predicted occurrence of SVs across conventionally bred and wild varieties of agricultural crops, including maize, rice, grapevine, rapeseed and tomato (Chia *et al*., 2012; Wang *et al*., 2018; Zhou *et al*., 2019; Fuentes *et al*., 2019; Alonge *et al*., 2020; Chawla *et al.*, 2020; Huang & Rieseberg, 2020; Orantes-Bonilla *et al*., 2022; Yildiz *et al*., 2023).

In conclusion, although predominantly smaller insertions are detected after use of random mutagenesis techniques, larger structural rearrangements involving insertions are also not uncommon.

3. Number of mutations introduced by random mutagenesis techniques

The number of mutations and mutated genes observed is dependent, amongst various factors, on the random mutagenesis technique used. In FN-irradiated rice, the average number of mutated genes per line was 31 (varying between seven and 147) and the number of mutations per line was on average 59 (varying between 28 and 78) (Li *et al.,* 2016a). In *Arabidopsis*, six FN-irradiated mutation lines were reported to display a number of mutations ranging between eight and 32 per line (Belfield *et al.,* 2012). Between 41 and 76 homozygous substitutions were found in 10 lines with a mutant phenotype in FN-irradiated soybean (Anderson *et al.,* 2016). As mentioned above, 1216 duplications and

deletions were induced by FN irradiation in a total of 264 soybean plants (averaging one segmental duplication, two to three homozygous deletions and one hemizygous deletion per individual) (Bolon *et al.,* 2014). In EMS-treated rice, per plant an average of 37 mutations that were deleterious for the gene's function was observed in a population of 72 individuals (the total number of mutations was more than 2700) (Henry *et al.,* 2014). On average, higher mutation densities are seen in polyploid species when using random mutagenesis techniques (Kurowska *et al.,* 2011).

4. Mutations as a result of other conventional breeding techniques

4.1. *In vitro* plant tissue/cell culture and clonal propagation

Genetic variation may result from stress factors during *in vitro* plant tissue culture, cell culture propagation (somaclonal variation) or from clonal propagation. This variation can be the basis for the development of new and improved cultivars, but it is not always desirable, e.g. in cases of *in vitro* cloning or germplasm preservation (Krishna *et al*., 2016).

In *in vitro* propagated rice, somaclonal variation in the form of Single Nucleotide Polymorphisms (SNPs) (substitutions) and indels was observed. The mutation rate of these regenerated rice lines was estimated at 1.74×10^{-6} base substitutions per site per generation (Miyao *et al*., 2012), which is higher than the estimated natural mutation rate in rice of $\sim 5.4 \times 10^{-8}$ per site per diploid genome per generation (Tang *et al*., 2018). Zhang *et al*. (2014) also identified extensive inheritable somaclonal genomic variation in rice tissue culture and estimated a mutation rate of 5×10^{-5} base substitutions per site.

Non-heritable somatic mutations can accumulate in clonal propagation of micropropagated crops (e.g. strawberry, banana, potato and coffee). More than 1 million Single Nucleotide Variants (SNVs, a single nucleotide change in the DNA) were found in a clonally propagated cannabis line, with variation seen between different tissues (Adamek *et al*., 2022). Larger structural genomic variations are also possible. For example, all analysed potatoes within a set regenerated from protoplasts displayed aneuploidy or structural chromosomal changes (Fossi *et al*., 2019). In addition, gene duplications and insertions of variable sizes can occur through transposon activity (Cerbin & Jiang, 2018).

The above genetic changes are sometimes intentionally induced: the chemical mutagen colchicine is commonly applied in conventional breeding for polyploidisation *in vitro* (Alemanno & Guiderdoni, 1994; Eng & Ho, 2019). In addition, several commercial varieties belonging to various species have been derived from somaclonal variation (Bhojwani & Dantu, 2013; Krishna *et al*., 2016).

4.2. Natural mutation rate and inter-cultivar variation

Ossowski *et al*. (2010) observed a natural mutation rate of 7.1×10^{-9} per site per generation in *A. thaliana*. A more recent study with this plant came to a similar conclusion with 6.95×10^{-9} single nucleotide mutations per site per generation for lines that went through 25 generations. The rate of occurrence of indels was lower at 1.30×10^{-9} per site per generation and deletions were more frequent and larger (excluding the seven deletions greater than 100 bp, the mean was 6.5 bp) than insertions (mean 3.9 bp) (Weng *et al*., 2019). In maize, the natural mutation rate was estimated at 2.17-3.87 x 10^{-8} per site per generation (Yang *et al*., 2017) and in rice $\sim 5.4 \times 10^{-8}$ per site per diploid genome per generation (Tang *et al*., 2018). Analyses of SNPs and indels showed that these were common in twelve analysed maize lines: SNPs and indels occurred on average every 73 and 309 bp, respectively (Vroh Bi *et al*., 2005).

Genomic structural variation is also found in polyploid crops and can take the form of presence-absence variation, copy-number variation and homoeologous exchanges (Schiessl *et al*., 2018). Although the natural mutation rate is lower than the rate obtained through induced random

mutagenesis, the amount of inter-cultivar variation already available is extensive. Anderson *et al*. (2016) examined the genomic variation in soybean cultivars and mutagenized plants. The inter-cultivar variation extending to over 1 million SNPs was far greater than the variation seen amongst FN and *Agrobacterium*-transformed plants, which led to less than 100 single nucleotide substitutions genome-wide. Other examples include variation among elite maize inbred lines (Lai *et al*., 2010), structural variation in rice (Fuentes *et al*., 2019) and variation in US wheat varieties (Sthapit *et al*., 2022).

5. Gene introgression

Whole Genome Sequencing (WGS) can be used to identify the genetic diversity present in a species' gene pool which then can be utilized to introgress genetic regions of interest into elite cultivars by crop improvement programs (Tao *et al*., 2019).

5.1. Resistance breeding

Resistance breeding is used for the development of new cultivars resistant to pathogens by introgressing *Resistance* genes (*R* genes) from wild germplasm into agricultural varieties (Dangl *et al*., 2013). This is often a time-consuming task, further complicated by the lack of knowledge on a large proportion of plant genetic diversity that, to date, remains uncharacterized. Next-generation sequencing (NGS) and high-throughput genotyping technologies (HTGT) can contribute to unveiling new *R* genes (Sánchez-Martín *et al.*, 2019). Introgressing *R* genes into elite cultivars can be time consuming for some crops such as those that are usually vegetatively propagated (e.g. potato and banana) or trees (e.g. apple and citrus). In potato, it can take up to 50 years to introgress resistance into a new variety (Haverkort *et al*., 2009). Furthermore, to prevent resistance breaking in the field, multiple *R* genes would need to be introgressed (Dangl *et al*., 2013). The stacking of *R* genes through resistance breeding ("gene pyramiding") has been demonstrated in sexually propagated crops such as tomato, wheat, and pepper (Fuchs, 2017). For example, marker assisted breeding was used to cross two grapevine cultivars carrying one *R* gene each to generate a new cultivar with two *R* genes (Eibach *et al*., 2007). Similar successful attempts have been undertaken, *inter alia*, in rice for bacterial leaf blight resistance (Suh *et al*., 2013), in maize for different virus resistances (Zambrano *et al*., 2014), in barley for resistance against various pathogens (Friedt & Ordon 2007) and in wheat for powdery mildew (Liu *et al*., 2008) and stem rust resistance (Liu *et al*., 2020).

5.2. Examples of introgression of other traits

Hybridization and mutation breeding are two techniques that can be used for breeding soybean varieties with high protein content (Guo *et al*., 2022). Several introgression lines carrying quantitative trait loci (QTLs) for productivity, biotic and abiotic stress tolerance can be used for breeding programs (Ashikari & Matsuoka, 2006; Lippman *et al*., 2007). Abiotic tolerance traits have also been introgressed in commercial varieties, for example in sunflower by hybridising two North American species (Whitney *et al*., 2010). Traits related to drought tolerance have also been introgressed in cereal crops such as rice (Dharmappa *et al*., 2019), wheat (Placido *et al*., 2013) and chickpea (Bharadwaj *et al*., 2021).

6. Modifications that are difficult to obtain by conventional breeding techniques

Through conventional breeding, new or improved cultivars are made by stacking genes or making new genomic combinations (Prohens, 2011; Bradshaw, 2017). Random mutagenesis has further advanced conventional breeding, by inducing mutations in agronomical interesting crops, which can be later crossbred into elite cultivars. However, these techniques are more successful for some crops and genes than for others. Several examples are given below where conventional breeding is slower and/or less efficient compared to certain NGTs.

Gene stacking in vegetatively propagated crops is relatively difficult using conventional breeding techniques despite advances such as speed breeding, genotyping, marker-assisted selection and high-throughput phenotyping (Hickey *et al*., 2019; Hasan *et al*., 2021). Another factor making stacking of desirable traits less efficient is the possibility of linkage drag: the association and carry-over of undesirable traits with desirable ones (Wolter *et al*., 2019; Lee & Wang, 2020). Furthermore, the targeting of specific genes is not possible when using conventional mutagenesis techniques. When using these techniques large progeny populations and extensive screening are thus required. Nonaka *et al*. (2017) reported that, in an attempt to mutate the C-terminus of *GAD3* to increase the level of γ-aminobutyric acid (GABA) in tomato fruits, no such mutation was found among ~4500 lines resulting from EMS mutagenesis. Likewise, the targeting of multiple homologous genes or of genes responsible for polygenic traits, especially in polyploid species, can be difficult using conventional breeding techniques (Liu *et al*., 2022; Martínez-Fortún *et al*. 2022). For example, *MLO* is a well-known pathogen *susceptibility* gene that leads to enhanced resistance to powdery mildew when knocked out (Jørgensen, 1992). *Mlo* mutants have since also been naturally found or induced through random mutagenesis in crops such as cucumber, pea and tomato (Kusch & Panstruga 2017). CRISPR/Cas was used to engineer the same trait in bread wheat, since no spontaneous or induced *mlo* mutants had been reported, probably due to the presence of three different *MLO* homoeoalleles (Wang *et al*., 2014). Since then, the same results were obtained by Targeting Induced Lesions IN Genomes (TILLING) and combining the mutations by crosses (Acevedo-Garcia *et al*., 2017). Conventional breeding techniques can therefore be used to obtain similar results as can be achieved by the application of certain NGTs. However, on average the conventional breeding techniques are less efficient, require more resources and take longer than targeted mutagenesis techniques. Even more so when a large number of genes need to be altered to achieve the desired trait.

An example of multiple targeted gene knockouts is the low-gluten wheat that was obtained by CRISPR-Cas editing of several homologs in the α-gliadin gene family (Sánchez-León *et al*., 2018). Low-gluten products have been obtained through conventional breeding, such as low-gluten barley and wheat by combining recessive alleles or deletion lines (Tanner *et al*., 2016; Van den Broeck *et al*., 2009). Compared to conventional breeding however, the use of NGTs for multiplex targeted gene editing appears to be more efficient and requires less back-crosses (Nogué *et al*., 2016).

7. Conclusions

In this analysis, the most frequently reported mutations resulting from random mutagenesis techniques are SBSs, followed by deletions and lastly insertions. Although the mutation frequency when using random mutagenesis is higher compared to natural mutation rates, it is lower than the total accumulated number of SNPs naturally occurring between different cultivars (Anderson *et al*., 2016) or the number of genetic mutations resulting from tissue culture (Zhang *et al*., 2014), clonal propagation (Adamek *et al*., 2022) or protoplast regeneration (Fossi *et al*., 2019).

Larger deletions, translocations, inversions and genome duplications also occur naturally; these mutations depend on a variety of biological processes (e.g. homologous vs. non homologous DNA repair, or transposon activity (Xiao *et al*., 2008; Anderson *et al*., 2019)). So far, they have been reported to occur less frequently than smaller mutations, but the improvement of detection methods (i.e. long-read sequencing) has started to unveil higher rates than previously estimated (Chia *et al*., 2012; Wang *et al*., 2018; Zhou *et al*., 2019; Fuentes *et al*., 2019; Alonge *et al*., 2020; Chawla *et al*., 2020; Yildiz *et al*., 2023; Lemay *et al*., 2022; Zhang *et al*., 2022). The occurrence of larger genome modifications can be enhanced using conventional breeding techniques (Custers *et al*. 2019; Martínez-Fortún *et al*. 2022). Introgression of cisgenes is also possible and broadly performed by conventional breeding methods. Finally, the average number of mutations per gene and the number of mutated lines observed with conventional breeding methods is variable and dependent on the technique and the reproductive capability of the plant material.

In certain cases, NGTs can produce genetic modifications that are difficult to obtain by conventional breeding techniques: 1) "Gene pyramiding" is common for sexually propagated crops, but it is more challenging for crops with a low regenerative potential or a long generation time (e.g. trees). Cisgenesis using NGTs can greatly improve efficiency and reduce breeding time compared to conventional "gene pyramiding". 2) Targeting of multiple homologous genes is efficient using NGTs, but it is impractical or extremely difficult using random mutagenesis, as it involves screening of large progeny populations.

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