This draft has not been adopted or endorsed by the European Commission. Any views expressed are the preliminary views of the Commission services and may not in any circumstances be regarded as stating an official position of the Commission. The information transmitted is intended only for the Member State or entity to which it is addressed for discussion.

Potential criteria to determine whether a plant obtained by targeted mutagenesis or cisgenesis could also occur naturally or be produced by conventional breeding techniques

Introduction

In the context of the impact assessment of a legislation for plants produced by targeted mutagenesis and cisgenesis, the public consultation enquired about possible criteria to assess whether a plant obtained by targeted mutagenesis and cisgenesis could also occur naturally or be produced by conventional breeding techniques (**equivalence criteria**) and the targeted consultation provided an initial comprehensive indicative list of criteria as a working basis for the impact assessment analysis. This indicative list of potential equivalence criteria was developed taking into account relevant work of the JRC and EFSA, available scientific literature and the analysis of similar criteria used in third country legislation.

Various comments on these possible equivalence criteria were received in the consultations and work continues on them, taking into account available scientific evidence in the literature as well as the feedback received during the consultation.

Purpose of this note

This note serves as a basis for discussion with Member States on potential equivalence criteria to assess whether a plant obtained by targeted mutagenesis and cisgenesis could also occur naturally or be produced by conventional breeding techniques. It does not prejudge in any way a future policy decision based on the impact assessment on a legislative proposal for plants produced by targeted mutagenesis and cisgenesis.

Potential equivalence criteria

A thorough analysis of the literature confirmed that targeted mutagenesis and cisgenesis techniques can lead to genetic modifications that are similar to mutations occurring naturally or resulting from conventional breeding techniques (including random mutagenesis techniques). These mutations include **substitutions, insertions and deletions** of nucleotides in the DNA. Such mutations are commonly observed with conventional breeding techniques and are of **variable sizes**: substitutions and insertions introduced by conventional breeding techniques are often of limited size (although large substitutions and insertions can also occur), while both small and large deletions are frequently observed. Insertions of cisgenes or part of cisgenes are also possible through natural crossing or conventional breeding; however, the insertion of part of a cisgene is generally only possible when it takes place in its natural location. As regards the **number of modifications** introduced by conventional breeding techniques, the literature shows that the number of mutated DNA sequences is also variable (see technical annex for the detailed analysis).

It seems appropriate that the criteria would be product rather than technique-based, reflecting the majority scientific view that the risk profile is mainly dependent on the characteristics of the product. Product-based criteria would also be more future-proof compared to technique-based criteria.

As shown by the scientific literature analysis, conventional breeding techniques can lead to a high number of modifications. However, the likelihood that certain combinations of modifications occur by conventional methods may be low. To take this into account, a threshold for the combinations of different modifications might be considered.

Based on the above, the following type of criteria could be considered:

1. **Targeted mutagenesis**
    a. Substitution and insertion of a limited size.
    b. Deletion of any size.
2. **Cisgenesis**
    a. Targeted insertion of a complete cisgene.
    b. Targeted substitution of a part of a cisgene in its natural location.
3. **Combinations of the above modifications**: Up to a pre-defined threshold.

For a possible application of the criteria, all modifications would have to fulfil at least one of the criteria under 1 or 2. In addition, they would have always to fulfil criterion 3.

Member State experts are invited to provide their views on the potential criteria above and the analysis in the annex. A discussion will take place at the forthcoming Joint Working Group meeting of GMO competent authorities of 9 February 2023 and if appropriate may be followed by further exchanges of views on the matter.

Annex

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

Introduction

Targeted mutagenesis and cisgenesis techniques can lead to genetic modifications that are similar to mutations that are generated following the application of certain conventional breeding techniques, such as random mutagenesis, or that occur spontaneously in nature. The mutations induced by random mutagenesis techniques (e.g. ethyl methanesulfonate (EMS), gamma rays, fast-neutron irradiation, etc.) include **nucleotide substitutions, insertions and deletions** of various sizes. We analysed the scientific literature regarding the size and occurrence of these type of mutations, as well as the number of mutated genes. We mainly focused on random mutagenesis techniques such as irradiation and EMS when determining the size and occurrence of mutations. These mutations are comparable to mutations derived from New Genomic Techniques (NGTs), in which breaks are induced in the DNA and edits result from imperfections in the natural DNA repair mechanism of plants (Pacher and Puchta 2017). 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 cultivars, cumulated over time, is much larger than the number of mutations induced by random mutagenesis (Anderson et al. 2016).

Type and size of mutations caused by random mutagenesis techniques

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 fast-neutron (FN) irradiation in rice (52.6% of observed mutations) (Li et al. 2016a). The majority of mutations in carbon 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 on the genome (Li et al. 2016b). Also, the application of EMS in Arabidopsis, soybean and rice for random mutagenesis leads to predominantly transition mutations (>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.

Deletions

Although SBSs were most abundant, deletions affected the largest number of mutated genes in FN mutated rice (71.5%) (Li et al. 2016a). In FN mutated *A. thaliana* lines, deletions sizes were as high as 12 kB, but the majority of the deletions were between 0-4 kb in size (58.3%) (Li et al. 2001). The majority of indels in FN mutated rice lines were small (≤10 bp) deletion and insertion events (73.2%), although large deletions also occurred and affected several genes (e.g. 49 genes were disrupted by a 300 kb deletion in one line) (Li et al. 2016a). Large deletions were also observed in 264 analysed FN irradiated soybean lines, where an average of 2-3 homozygous deletions (>500 bp) per line was detected (Bolon et al. 2014). In five FN irradiated common bean plants large deletions that ranged in size from 40 bp to 43,000 bp were found (O´Rourke et al. 2013). The most frequent mutation type in 24 individuals with a mutant phenotype after gamma irradiation of rice were small deletions (1-16 bp; 62.5%) (Morita et al. 2009). The majority of the deletions in carbon-ion irradiated dry seed and seedlings of *A. thaliana* were <50 bp (95%

in dry seed, and 91.5% in seedlings), but larger deletions (>1 kb) were also observed (Hase et al. 2018). Single base deletions were most frequently observed (36%) in FN irradiated *Arabidopsis*, but again larger deletions were also seen (up to 7.2 kb) (Belfield et al. 2012). The majority of the deletions (97%) were <56 bp in length (Belfield et al. 2012). Large deletions were also seen in characterised deletion mutant alleles, derived from random mutagenesis techniques (FN bombardment) (Li and Zhang 2002). In conclusion, although small deletions seem to be more abundant when using random mutagenesis techniques, large deletions also frequently occur.

Insertions

In FN irradiated rice, most insertions were 1 bp long (69%). Insertions of 2-6 bp were also observed (27%), while insertions of >10 bp (up to 26 bp) were rare (Li et al. 2016a, Supplemental material "insertions"). 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 rest ranged between 3 and 46 bp (Hase et al. 2018, Table S5). In Belfield et al. (2012), only +1 bp insertions were seen in six FN irradiated mutated Arabidopsis lines. Although insertions are less reported than deletions, they do occur and the length of the insertions is usually limited. Moreover, next to insertions, duplications of genic regions are also possible: in 264 FN-treated soybean plants, on average one segmental duplication (with a mean size of >2 Mb) was identified per mutant line (Bolon et al. 2014).

Number of mutations introduced by random mutagenesis techniques

The number of mutations and mutated genes observed are dependent on the random mutagenesis technique used. In FN irradiated rice, the average number of mutated genes per line was 31 (varying between 7 and 147) and the number of mutations per line was on average 59 (Li et al. 2016a). In another paper, where six mutation lines of FN irradiated *Arabidopsis* were analysed, the number of mutations ranged between eight and 32 per line (Belfield et al. 2012). In EMS treated rice, an average of 37 deleterious gene mutations per plant was observed in a population of 72 individuals (the total number of mutations was >2700) (Henry et al. 2014). Between 41 and 76 homozygous substitutions were found in 10 lines with a mutant phenotype in FN irradiated soybean (Anderson et al. 2016, Table S3). As mentioned above, 1216 duplications and deletions were induced by FN irradiation in a total of 264 soybean plants (averaging one segmental duplication, 2-3 homozygous deletions, and 1 hemizygous deletion per individual (Bolon et al. 2014)). On average, higher mutation densities are seen in polyploid species when using random mutagenesis techniques (Kurowska et al. 2011).

Mutations as a result of other conventional breeding techniques

In vitro plant tissue/cell culture and clonal propagation

Genetic variation may result from stress factors during *in vitro* plant tissue or cell culture propagation (somaclonal variation) or from clonal propagation. This variation can be a source of mutations for the development of new and improved cultivars, but it is not always desired, 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) and indels was observed. The mutation rate of these regenerated rice lines was estimated as 1.74×10^{-6} base substitutions per site per generation (Miyao et al. 2012). 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). In Adamek et al. (2022) 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.

Larger structural genomic variations are also possible. For example, all analysed potatoes regenerated from protoplasts displayed aneuploidy or structural chromosomal changes (Fossi et al. 2019). In addition, gene duplications and new gene insertions can occur through transposon activity (Cerbin and Jiang 2018). These changes are sometimes intentionally induced: the chemical mutagen colchicine is commonly applied *in vitro* for polyploidisation in conventional breeding (Alemanno and Guiderdoni 1994; Eng and Ho 2019). Several commercial varieties from various species have been derived from somaclonal variation (Bhojwani and Dantu 2013; Krishna et al. 2016).

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 came to a similar conclusion with 6.95×10^{-9} per site per generation of single nucleotide mutations for lines that went through 25 generations in the lab (Weng et al. 2019). The rate of indels was lower (1.30×10^{-9} per site per generation) and deletions were more frequent and larger (excluding deletions >100 bp, the mean was 6.5 bp) than insertions (mean 3.9 bp) (Weng et al. 2019). In maize, the natural substitution rate was estimated as 2.17×10^{-8} and 3.87×10^{-8} per site per generation (Yang et al. 2017). Analysis of SNPs and indels showed that these were common in 12 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 homeologous 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 present is extensive. Anderson et al. (2016) examined the genomic variation in soybean cultivars and mutagenized plants. The inter-cultivar variation (> 1 million SNPs) was far more extensive than the variation seen in FN and *Agrobacterium*-transformed plants (<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 genetic diversity in US wheat varieties (Sthapit et al. 2022).

Gene introgression

Whole Genome Sequencing (WGS) can be used to identify the genetic diversity present in a species, and this can be utilized in crop improvement programs by introgressing genetic regions into elite cultivars (Tao et al. 2019).

Resistance breeding

Resistance breeding is used for the development of new cultivars resistant to pathogens by introgressing *Resistance* genes (*R* genes) from wild germplasm (Dangl et al. 2013). This is often a time-consuming task, further limited by the large pool of genetic plant diversity that remains uncharacterized (Sánchez-Martín et al. 2019). 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, a cross was made between two grapevine cultivars carrying one *R* gene each to generate a new cultivar with two *R* genes using marker assisted breeding (Eibach et al. 2007). Similar successful attempts have been done in rice for bacterial leaf blight resistance (Suh et al. 2013), in maize for virus resistance (Zambrano et al. 2014), in barley for disease resistance breeding (Friedt and Ordon 2007), and in wheat for powdery mildew (Liu et al. 2008) and stem rust resistance (Liu et al. 2020) to name a few.

Examples of introgression of other traits

Hybridization and mutation breeding are two techniques that can be used in breeding soybean to create varieties with high protein content (Guo et al. 2022). Several introgression lines carrying quantitative trait loci (QTLs) in e.g. rice and tomato can be used for breeding programs (Ashikari and Matsuoka 2006; Lippman et al. 2007). Abiotic tolerance traits have been introgressed in sunflower by hybridising two North American sunflower species (Whitney et al. 2010). Traits related to drought tolerance have also been introgressed in e.g. rice (Dharmappa et al. 2019) and wheat (Placido et al. 2013).

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 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 of desirable with undesirable traits (Wolter et al. 2019; Lee and Wang, 2020). Furthermore, the targeting of specific genes is not possible when using conventional mutagenesis techniques, hence requiring large progeny populations and extensive screening. Nonaka et al. (2017) reported that, in an attempt to mutate the C-terminal of *GAD3* to increase the level of GABA in fruits, no such mutation was found in an EMS population of ~4500 lines. Likewise, the targeting of homologous genes especially in polyploidy species can be difficult using conventional breeding techniques (Liu 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 and Panstruga 2017). NGTs (CRISPR/Cas) has been used in bread wheat since no spontaneous or induced *mlo* mutants had been reported, probably due to the presence of three *MLO* homoeoalleles (Wang et al. 2014). Since then, the same results were obtained by TILLING and combining the mutations by crosses (Acevedo-Garcia et al. 2017). Conventional breeding techniques can therefore be used to obtain similar results to NGT derived products, but it is less efficient, requires more resources and takes longer. Even more so when an increasing number of genes or difficult to edit genes are targeted.

An example of multiple gene knockouts is the low-gluten wheat that was obtained by CRISPR-Cas editing by targeting homologs of the α-gliadin gene family (Sánchez-León et al. 2018). Similar 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).

Conclusions

The most frequent mutations resulting from random mutagenesis techniques are SBSs, followed by deletions, and lastly short insertions. Although the mutation frequency when using random mutagenesis is higher compared to the natural mutation rate, it is lower than the accumulated number of SNPs naturally seen between 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; they are less common and crop-dependent (e.g. during the repair of DNA breaks, or due to (retro-) transposons (Xiao et al. 2008; Anderson et al. 2019)), but can be enhanced when using conventional breeding techniques (Custers et al. 2019; Martínez-Fortún et al. 2022). Introgression of genes is also possible by conventional breeding methods. Finally, the number of mutations per gene and mutated line observed with conventional breeding methods is quite variable and dependent on the technique and the reproduction 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 more challenging for crops with a low regenerative potential or long generation time. Cisgenesis using NGTs can greatly improve efficiency and reduce breeding time. 2) Targeting of multiple homologous genes is efficient using NGTs, but is impractical or extremely difficult using random mutagenesis, which involves screening of large progeny populations.

References:

1. Acevedo-Garcia, J., Spencer, D., Thieron, H., Reinstädler, A., Hammond-Kosack, K., Phillips, A. L., & Panstruga, R. (2017). mlo-based powdery mildew resistance in hexaploid bread wheat generated by a non-transgenic TILLING approach. *Plant biotechnology journal*, *15*(3), 367–378. https://doi.org/10.1111/pbi.12631
2. Adamek, K., Jones, A. M. P., & Torkamaneh, D. (2022). Accumulation of somatic mutations leads to genetic mosaicism in cannabis. *The plant genome*, *15*(1), e20169. https://doi.org/10.1002/tpg2.20169
3. Alemanno, L., & Guiderdoni, E. (1994). Increased doubled haploid plant regeneration from rice (Oryza sativa L.) anthers cultured on colchicine-supplemented media. *Plant cell reports*, *13*(8), 432–436. https://doi.org/10.1007/BF00231961Anderson, J. E., Michno, J. M., Kono, T. J., Stec, A. O., Campbell, B. W., Curtin, S. J., & Stupar, R. M. (2016). Genomic variation and DNA repair associated with soybean transgenesis: a comparison to cultivars and mutagenized plants. *BMC biotechnology*, *16*(1), 41. https://doi.org/10.1186/s12896-016-0271-z
4. Anderson, S. N., Stitzer, M. C., Brohammer, A. B., Zhou, P., Noshay, J. M., O'Connor, C. H., Hirsch, C. D., Ross-Ibarra, J., Hirsch, C. N., & Springer, N. M. (2019). Transposable elements contribute to dynamic genome content in maize. *The Plant journal : for cell and molecular biology*, *100*(5), 1052–1065. https://doi.org/10.1111/tpj.14489
5. Ashikari, M., & Matsuoka, M. (2006). Identification, isolation and pyramiding of quantitative trait loci for rice breeding. *Trends in plant science*, *11*(7), 344–350. https://doi.org/10.1016/j.tplants.2006.05.008
6. Belfield, E. J., Gan, X., Mithani, A., Brown, C., Jiang, C., Franklin, K., Alvey, E., Wibowo, A., Jung, M., Bailey, K., Kalwani, S., Ragoussis, J., Mott, R., & Harberd, N. P. (2012). Genome-wide analysis of mutations in mutant lineages selected following fast-neutron irradiation mutagenesis of Arabidopsis thaliana. *Genome research*, *22*(7), 1306–1315. https://doi.org/10.1101/gr.131474.111
7. Bhojwani, S. & Dantu, P. (2013). Plant Tissue Culture: An Introductory Text. 10.1007/978-81-322-1026-9_19
8. Bolon, Y. T., Stec, A. O., Michno, J. M., Roessler, J., Bhaskar, P. B., Ries, L., Dobbels, A. A., Campbell, B. W., Young, N. P., Anderson, J. E., Grant, D. M., Orf, J. H., Naeve, S. L., Muehlbauer, G. J., Vance, C. P., & Stupar, R. M. (2014). Genome resilience and prevalence of segmental duplications following fast neutron irradiation of soybean. *Genetics*, *198*(3), 967–981. https://doi.org/10.1534/genetics.114.170340

9. Bradshaw, J. E. (2017). Plant breeding: past, present and future. Euphytica. 213. 10.1007/s10681-016-1815-y
10. Cerbin, S., & Jiang, N. (2018). Duplication of host genes by transposable elements. *Current opinion in genetics & development*, *49*, 63–69. https://doi.org/10.1016/j.gde.2018.03.005
11. Cooper, J. L., Till, B. J., Laport, R. G., Darlow, M. C., Kleffner, J. M., Jamai, A., El-Mellouki, T., Liu, S., Ritchie, R., Nielsen, N., Bilyeu, K. D., Meksem, K., Comai, L., & Henikoff, S. (2008). TILLING to detect induced mutations in soybean. *BMC plant biology*, *8*, 9. https://doi.org/10.1186/1471-2229-8-9
12. Custers, R., Casacuberta, J. M., Eriksson, D., Sági, L., & Schiemann, J. (2019). Genetic Alterations That Do or Do Not Occur Naturally; Consequences for Genome Edited Organisms in the Context of Regulatory Oversight. *Frontiers in bioengineering and biotechnology*, *6*, 213. https://doi.org/10.3389/fbioe.2018.00213
13. Dangl, J. L., Horvath, D. M., & Staskawicz, B. J. (2013). Pivoting the plant immune system from dissection to deployment. *Science (New York, N.Y.)*, *341*(6147), 746–751. https://doi.org/10.1126/science.1236011
14. Dharmappa, P. M., Doddaraju, P., Malagondanahalli, M. V., Rangappa, R. B., Mallikarjuna, N. M., Rajendrareddy, S. H., Ramanjinappa, R., Mavinahalli, R. P., Prasad, T. G., Udayakumar, M., & Sheshshayee, S. M. (2019). Introgression of Root and Water Use Efficiency Traits Enhances Water Productivity: An Evidence for Physiological Breeding in Rice (Oryza sativa L.). *Rice (New York, N.Y.)*, *12*(1), 14. https://doi.org/10.1186/s12284-019-0268-z
15. Eibach, R., Zyprian, E., Welter, L., & Topfer, R. (2007). The use of molecular markers for pyramiding resistance genes in grapevine breeding. *VITIS-GEILWEILERHOF-*, *46*(3), 120.
16. Eng, W.H., & Ho, W. (2019). Polyploidization using colchicine in horticultural plants: A review. *Scientia Horticulturae*.
17. Fossi, M., Amundson, K., Kuppu, S., Britt, A., & Comai, L. (2019). Regeneration of *Solanum tuberosum* Plants from Protoplasts Induces Widespread Genome Instability. *Plant physiology*, *180*(1), 78–86. https://doi.org/10.1104/pp.18.00906
18. Friedt, W., & Ordon, F. (2007). Molecular markers for gene pyramiding and disease resistance breeding in barley. *Genomics-assisted crop improvement*, 81-101.
19. Fuchs M. (2017). Pyramiding resistance-conferring gene sequences in crops. *Current opinion in virology*, *26*, 36–42. https://doi.org/10.1016/j.coviro.2017.07.004
20. Fuentes, R. R., Chebotarov, D., Duitama, J., Smith, S., De la Hoz, J. F., Mohiyuddin, M., Wing, R. A., McNally, K. L., Tatarinova, T., Grigoriev, A., Mauleon, R., & Alexandrov, N. (2019). Structural variants in 3000 rice genomes. *Genome research*, *29*(5), 870–880. https://doi.org/10.1101/gr.241240.118
21. Greene, E. A., Codomo, C. A., Taylor, N. E., Henikoff, J. G., Till, B. J., Reynolds, S. H., Enns, L. C., Burtner, C., Johnson, J. E., Odden, A. R., Comai, L., & Henikoff, S. (2003). Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in Arabidopsis. *Genetics*, *164*(2), 731–740. https://doi.org/10.1093/genetics/164.2.731
22. Guo, B., Sun, L., Jiang, S., Ren, H., Sun, R., Wei, Z., Hong, H., Luan, X., Wang, J., Wang, X., Xu, D., Li, W., Guo, C., & Qiu, L. J. (2022). Soybean genetic resources contributing to sustainable protein production. *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik*, *135*(11), 4095–4121. https://doi.org/10.1007/s00122-022-04222-9
23. Hasan, N., Choudhary, S., Naaz, N. *et al.* Recent advancements in molecular marker-assisted selection and applications in plant breeding programmes. *J Genet Eng Biotechnol* **19**, 128 (2021). https://doi.org/10.1186/s43141-021-00231-1
24. Hase, Y., Satoh, K., Kitamura, S., & Oono, Y. (2018). Physiological status of plant tissue affects the frequency and types of mutations induced by carbon-ion irradiation in Arabidopsis. *Scientific reports*, *8*(1), 1394. https://doi.org/10.1038/s41598-018-19278-1
25. Haverkort, A.J., Struik, P.C., Visser, R.G.F. *et al.* Applied Biotechnology to Combat Late Blight in Potato Caused by *Phytophthora Infestans* . *Potato Res.* **52**, 249–264 (2009). https://doi.org/10.1007/s11540-009-9136-3
26. Henry, I. M., Nagalakshmi, U., Lieberman, M. C., Ngo, K. J., Krasileva, K. V., Vasquez-Gross, H., Akhunova, A., Akhunov, E., Dubcovsky, J., Tai, T. H., & Comai, L. (2014). Efficient Genome-Wide Detection and Cataloging of EMS-Induced Mutations Using Exome Capture and Next-Generation Sequencing. *The Plant cell*, *26*(4), 1382–1397. https://doi.org/10.1105/tpc.113.121590
27. Hickey, L. T., N Hafeez, A., Robinson, H., Jackson, S. A., Leal-Bertioli, S. C. M., Tester, M., Gao, C., Godwin, I. D., Hayes, B. J., & Wulff, B. B. H. (2019). Breeding crops to feed 10 billion. *Nature biotechnology*, *37*(7), 744–754. https://doi.org/10.1038/s41587-019-0152-9
28. Jørgensen, I.H. Discovery, characterization and exploitation of Mlo powdery mildew resistance in barley. *Euphytica* **63**, 141–152 (1992). https://doi.org/10.1007/BF00023919
29. Krishna, H., Alizadeh, M., Singh, D., Singh, U., Chauhan, N., Eftekhari, M., & Sadh, R. K. (2016). Somaclonal variations and their applications in horticultural crops improvement. *3 Biotech*, *6*(1), 54. https://doi.org/10.1007/s13205-016-0389-7

30. Kurowska, M., Daszkowska-Golec, A., Gruszka, D., Marzec, M., Szurman, M., Szarejko, I., & Maluszynski, M. (2011). TILLING: a shortcut in functional genomics. *Journal of applied genetics*, *52*(4), 371–390. https://doi.org/10.1007/s13353-011-0061-1
31. Kusch, S., & Panstruga, R. (2017). mlo-Based Resistance: An Apparently Universal "Weapon" to Defeat Powdery Mildew Disease. *Molecular plant-microbe interactions : MPMI*, *30*(3), 179–189. https://doi.org/10.1094/MPMI-12-16-0255-CR
32. Lai, J., Li, R., Xu, X., Jin, W., Xu, M., Zhao, H., Xiang, Z., Song, W., Ying, K., Zhang, M., Jiao, Y., Ni, P., Zhang, J., Li, D., Guo, X., Ye, K., Jian, M., Wang, B., Zheng, H., Liang, H., … Wang, J. (2010). Genome-wide patterns of genetic variation among elite maize inbred lines. *Nature genetics*, *42*(11), 1027–1030. https://doi.org/10.1038/ng.684
33. Lee, K., & Wang, K. (2020). Level up to chromosome restructuring. *Nature plants*, *6*(6), 600–601. https://doi.org/10.1038/s41477-020-0669-4
34. Li, X., Song, Y., Century, K., Straight, S., Ronald, P., Dong, X., Lassner, M., & Zhang, Y. (2001). A fast neutron deletion mutagenesis-based reverse genetics system for plants. *The Plant journal: for cell and molecular biology*, *27*(3), 235–242. https://doi.org/10.1046/j.1365-313x.2001.01084.x
35. Li, X., & Zhang, Y. (2002). Reverse genetics by fast neutron mutagenesis in higher plants. *Functional & integrative genomics*, *2*(6), 254–258. https://doi.org/10.1007/s10142-002-0076-0
36. Li, G., Chern, M., Jain, R., Martin, J. A., Schackwitz, W. S., Jiang, L., Vega-Sánchez, M. E., Lipzen, A. M., Barry, K. W., Schmutz, J., & Ronald, P. C. (2016a). Genome-Wide Sequencing of 41 Rice (Oryza sativa L.) Mutated Lines Reveals Diverse Mutations Induced by Fast-Neutron Irradiation. *Molecular plant*, *9*(7), 1078–1081. https://doi.org/10.1016/j.molp.2016.03.009
37. Li, S., Zheng, Y. C., Cui, H. R., Fu, H. W., Shu, Q. Y., & Huang, J. Z. (2016b). Frequency and type of inheritable mutations induced by γ rays in rice as revealed by whole genome sequencing. *Journal of Zhejiang University. Science. B*, *17*(12), 905–915. https://doi.org/10.1631/jzus.B1600125
38. Lippman, Z. B., Semel, Y., & Zamir, D. (2007). An integrated view of quantitative trait variation using tomato interspecific introgression lines. *Current opinion in genetics & development*, *17*(6), 545–552. https://doi.org/10.1016/j.gde.2007.07.007
39. Liu, J., Liu, D., Tao, W., Li, W., Wang, S., Chen, P., Cheng, S. and Gao, D. (2008), Molecular marker-facilitated pyramiding of different genes for powdery mildew resistance in wheat. Plant Breeding, 119: 21-24. https://doi.org/10.1046/j.1439-0523.2000.00431.x
40. Liu, T., Fedak, G., Zhang, L., Zhou, R., Chi, D., Fetch, T., Hiebert, C., Chen, W., Liu, B., Liu, D., Zhang, H., & Zhang, B. (2020). Molecular Marker Based Design for Breeding Wheat Lines with Multiple Resistance and Superior Quality. *Plant disease*, *104*(10), 2658–2664. https://doi.org/10.1094/PDIS-02-20-0420-RE
41. Liu, X., Zhang, S., Jiang, Y., Yan, T., Fang, C., Hou, Q., Wu, S., Xie, K., An, X., & Wan, X. (2022). Use of CRISPR/Cas9-Based Gene Editing to Simultaneously Mutate Multiple Homologous Genes Required for Pollen Development and Male Fertility in Maize. *Cells*, *11*(3), 439. https://doi.org/10.3390/cells11030439
42. Martínez-Fortún, J., Phillips, D. W., & Jones, H. D. (2022). Natural and artificial sources of genetic variation used in crop breeding: A baseline comparator for genome editing. *Frontiers in genome editing*, *4*, 937853. https://doi.org/10.3389/fgeed.2022.937853
43. Miyao, A., Nakagome, M., Ohnuma, T., Yamagata, H., Kanamori, H., Katayose, Y., Takahashi, A., Matsumoto, T., & Hirochika, H. (2012). Molecular spectrum of somaclonal variation in regenerated rice revealed by whole-genome sequencing. *Plant & cell physiology*, *53*(1), 256–264. https://doi.org/10.1093/pcp/pcr172
44. Morita, R., Kusaba, M., Iida, S., Yamaguchi, H., Nishio, T., & Nishimura, M. (2009). Molecular characterization of mutations induced by gamma irradiation in rice. *Genes and Genetic Systems*, *84*(5), 361-370. https://doi.org/10.1266/ggs.84.361
45. Nogué, F., Mara, K., Collonnier, C., & Casacuberta, J. M. (2016). Genome engineering and plant breeding: impact on trait discovery and development. *Plant cell reports*, *35*(7), 1475–1486. https://doi.org/10.1007/s00299-016-1993-z
46. Nonaka, S., Arai, C., Takayama, M., Matsukura, C., & Ezura, H. (2017). Efficient increase of ɣ-aminobutyric acid (GABA) content in tomato fruits by targeted mutagenesis. *Scientific reports*, *7*(1), 7057. https://doi.org/10.1038/s41598-017-06400-y
47. O'Rourke, J. A., Iniguez, L. P., Bucciarelli, B., Roessler, J., Schmutz, J., McClean, P. E., Jackson, S. A., Hernandez, G., Graham, M. A., Stupar, R. M., & Vance, C. P. (2013). A re-sequencing based assessment of genomic heterogeneity and fast neutron-induced deletions in a common bean cultivar. *Frontiers in plant science*, *4*, 210. https://doi.org/10.3389/fpls.2013.00210
48. Ossowski, S., Schneeberger, K., Lucas-Lledó, J. I., Warthmann, N., Clark, R. M., Shaw, R. G., Weigel, D., & Lynch, M. (2010). The rate and molecular spectrum of spontaneous mutations in Arabidopsis thaliana. *Science (New York, N.Y.)*, *327*(5961), 92–94. https://doi.org/10.1126/science.1180677

49. Pacher, M., & Puchta, H. (2017). From classical mutagenesis to nuclease-based breeding - directing natural DNA repair for a natural end-product. *The Plant journal : for cell and molecular biology*, *90*(4), 819–833. https://doi.org/10.1111/tpj.13469

50. Placido, D. F., Campbell, M. T., Folsom, J. J., Cui, X., Kruger, G. R., Baenziger, P. S., & Walia, H. (2013). Introgression of novel traits from a wild wheat relative improves drought adaptation in wheat. *Plant physiology*, *161*(4), 1806–1819. https://doi.org/10.1104/pp.113.214262

51. Prohens J. (2011). Plant Breeding: A Success Story to be Continued Thanks to the Advances in Genomics. *Frontiers in plant science*, *2*, 51. https://doi.org/10.3389/fpls.2011.00051

52. Sánchez-León, S., Gil-Humanes, J., Ozuna, C. V., Giménez, M. J., Sousa, C., Voytas, D. F., & Barro, F. (2018). Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. *Plant biotechnology journal*, *16*(4), 902–910. https://doi.org/10.1111/pbi.12837

53. Sánchez-Martín, J., & Keller, B. (2019). Contribution of recent technological advances to future resistance breeding. *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik*, *132*(3), 713–732. https://doi.org/10.1007/s00122-019-03297-1

54. Schiessl, S., Katche, E., Ihien, E., Chawla, H. S., Harmeet, Mason, A. (2018). The role of genomic structural variation in the genetic improvement of polyploid crops. The Crop Journal. 7. 10.1016/j.cj.2018.07.006

55. Sthapit, S. R., Ruff, T. M., Hooker, M. A., & See, D. R. (2022). Population structure and genetic diversity of U.S. wheat varieties. *The plant genome*, *15*(2), e20196. https://doi.org/10.1002/tpg2.20196

56. Suh, J. P., Jeung, J. U., Noh, T. H., Cho, Y. C., Park, S. H., Park, H. S., Shin, M. S., Kim, C. K., & Jena, K. K. (2013). Development of breeding lines with three pyramided resistance genes that confer broad-spectrum bacterial blight resistance and their molecular analysis in rice. *Rice (New York, N.Y.)*, *6*(1), 5. https://doi.org/10.1186/1939-8433-6-5

57. Tanner, G. J., Blundell, M. J., Colgrave, M. L., & Howitt, C. A. (2016). Creation of the first ultra-low gluten barley (Hordeum vulgare L.) for coeliac and gluten-intolerant populations. *Plant biotechnology journal*, *14*(4), 1139–1150. https://doi.org/10.1111/pbi.12482

58. Tao, Y., Zhao, X., Mace, E., Henry, R., & Jordan, D. (2019). Exploring and Exploiting Pan-genomics for Crop Improvement. *Molecular plant*, *12*(2), 156–169. https://doi.org/10.1016/j.molp.2018.12.016

59. Van den Broeck, H. C., van Herpen, T. W., Schuit, C., Salentijn, E. M., Dekking, L., Bosch, D., Hamer, R. J., Smulders, M. J., Gilissen, L. J., & van der Meer, I. M. (2009). Removing celiac disease-related gluten proteins from bread wheat while retaining technological properties: a study with Chinese Spring deletion lines. *BMC plant biology*, *9*, 41. https://doi.org/10.1186/1471-2229-9-41

60. Vroh Bi, I., McMullen, M. D., Sanchez-Villeda, H. E. C. T. O. R., Schroeder, S. T. E. V. E., Gardiner, J. A. C. K., Polacco, M., ... & Coe, E. H. (2006). Single nucleotide polymorphisms and insertion–deletions for genetic markers and anchoring the maize fingerprint contig physical map. *Crop Science*, *46*(1), 12-21.

61. Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., & Qiu, J. L. (2014). Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nature biotechnology*, *32*(9), 947–951. https://doi.org/10.1038/nbt.2969

62. Weng, M. L., Becker, C., Hildebrandt, J., Neumann, M., Rutter, M. T., Shaw, R. G., Weigel, D., & Fenster, C. B. (2019). Fine-grained analysis of spontaneous mutation spectrum and frequency in arabidopsis thaliana. *Genetics*, *211*(2), 703-714. https://doi.org/10.1534/genetics.118.301721

63. Whitney, K. D., Randell, R. A., & Rieseberg, L. H. (2010). Adaptive introgression of abiotic tolerance traits in the sunflower Helianthus annuus. *The New phytologist*, *187*(1), 230–239. https://doi.org/10.1111/j.1469-8137.2010.03234.x

64. Wolter, F., Schindele, P., & Puchta, H. (2019). Plant breeding at the speed of light: the power of CRISPR/Cas to generate directed genetic diversity at multiple sites. *BMC plant biology*, *19*(1), 176. https://doi.org/10.1186/s12870-019-1775-1

65. Xiao, H., Jiang, N., Schaffner, E., Stockinger, E. J., & Van Der Knaap, E. (2008). A retrotransposon-mediated gene duplication underlies morphological variation of tomato fruit. *science*, *319*(5869), 1527-1530.

66. Yang, N., Xu, X. W., Wang, R. R., Peng, W. L., Cai, L., Song, J. M., Li, W., Luo, X., Niu, L., Wang, Y., Jin, M., Chen, L., Luo, J., Deng, M., Wang, L., Pan, Q., Liu, F., Jackson, D., Yang, X., Chen, L. L., … Yan, J. (2017). Contributions of Zea mays subspecies mexicana haplotypes to modern maize. *Nature communications*, *8*(1), 1874. https://doi.org/10.1038/s41467-017-02063-5

67. Zambrano, J. L., Jones, M. W., Brenner, E., Francis, D. M., Tomas, A., & Redinbaugh, M. G. (2014). Genetic analysis of resistance to six virus diseases in a multiple virus-resistant maize inbred line. *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik*, *127*(4), 867–880. https://doi.org/10.1007/s00122-014-2263-5

68. Zhang, D., Wang, Z., Wang, N., Gao, Y., Liu, Y., Wu, Y., Bai, Y., Zhang, Z., Lin, X., Dong, Y., Ou, X., Xu, C., & Liu, B. (2014). Tissue culture-induced heritable genomic variation in rice, and their phenotypic implications. *PloS one*, *9*(5), e96879. https://doi.org/10.1371/journal.pone.0096879