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Unintended effects caused by techniques of new genetic engineering create a new quality of hazards and risks

Christoph Then with Lucy Sharratt

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Testbiotech is an independent institute for impact assessment in the field of genetic engineering. Our work is strictly based on scientific principles and evaluates the available information from the perspective of protecting health, the environment and nature. Testbiotech is free of any interests in the development, application and marketing of genetically engineered products. We are funded by private donations, public project and foundation funds.

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The Canadian Biotechnology Action Network (CBAN) brings together 16 groups to research, monitor and raise awareness about issues relating to genetic engineering in food and farming. CBAN members across Canada include farmer associations, environmental and social justice organizations, and regional coalitions of grassroots groups. CBAN is a project on the shared platform of the MakeWay Charitable Society. www.cban.ca

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Overview

In the European Union and Canada, there are ongoing debates about deregulating organisms derived from methods of new genetic engineering (New GE, also called genome editing or new genomic techniques).

Proposals to exempt genome editing from government regulation of genetically modified organisms (GMOs) largely rest on assumptions about similarities between genome editing and conventional plant breeding that are not supported by scientific findings. These assumptions have led to the impression that there are no new and specific risks caused by New GE as compared to conventional breeding.

Genome editing has the unprecedented power to make large parts of the genome accessible to change, by overriding the natural mechanisms of genome organization such as repair mechanisms or backup genes. Thereby, New GE techniques can cause pervasive changes in the genome of plants and animals, without inserting additional 'foreign' genes. These processes are also known to result in unintended effects, especially if 'gene scissors' (site directed nucleases or SDNs) such as CRISPR/Cas are applied. Both intended and unintended genetic changes can go far beyond what was seen in applications of previous methods. Many potential intended and unintended effects are specific to the techniques of New GE and may result in a new quality of risks that demand independent and mandatory risk assessment.

If these findings are overlooked in regulation, the introduction of New GE organisms will endanger ecosystems and food safety.

2. Differences in patterns of mutations used in conventional breeding compared to New GE

For the first time, genome editing makes large parts of the genome of many species accessible to change (via targeted mutations) (Kawall, 2019). The CRISPR/Cas techniques can override the natural mechanisms in genome organisation that protect essential genes (Belfield et al., 2018; Frigola et al., 2017; Halstead et al., 2020; Kawall, 2019; Monroe et al., 2022). As a result, novel genotypes and biological characteristics can emerge from applications of this technology. These observations are relevant to both intended and unintended effects.

For example, it is now shown that genes essential for the survival of species are more frequently repaired by natural mechanisms in the cells, compared to others, i.e. they are more protected from mutation (Huang & Li, 2018; Belfield et al., 2018; Monroe et al., 2022). In addition, both the structure of the chromosomes and the location of the genes influence the rate of mutations (Halstead et al., 2020; Monroe et al., 2022). Furthermore, gene duplications play a major role, in particular, in the genome of plants (Wendel et al., 2016; Gaines et al., 2019). Biological characteristics, such as herbicide resistance in weeds, can be fostered through gene duplication (Gaines et al., 2019) and backup functions established (Jones et al., 2017). These and other recent findings are challenging the classical evolutionary theory that mutations occur randomly, irrespective of their consequences for the organism (e.g. fitness costs).

On the other hand, if a site directed nuclease (e.g. CRISPR/Cas9 or TALENs) is designed to cut a specific DNA sequence, it will cut the same sequence again if the cell's own repair mechanism repairs it correctly. Such a nuclease will likely continue to cut until the intended incorrect repair is achieved and no more target sequence is available (Brinkman et al., 2018). Whilst this will result in high efficiency of cutting and mutating/changing of target sites, the same may be true for non-target sites with similar DNA sequences. Such changes would be unlike any other random mutations, as they would also override the cell's own protective

mechanisms, as well as potentially alter not just a single copy of a non-target gene, but several or all copies (depending on plant species and degree of ploidy). This is something that would not happen with conventional breeding, including with chemical or radiation-induced mutagenesis.

As a result, tools like CRISPR/Cas can prevent the cells from restoring the original function of the gene; they can also override other natural protection mechanisms (Kawall, 2019). In addition, CRISPR/Cas can also block the function of all the 'backup' copies of a target gene, of which there can be several in the genome of plants.

Thus, the techniques used for New GE can not only escape the boundaries of species, but also those of natural genome organisation (which impacts the rate and the distribution of mutations by repair mechanism and genomic factors such as several copies of one gene etc.). This persistence and overriding of the cell's own protective mechanisms makes genome editing a novel technology with novel capacities and consequences unlike any other. The resulting organisms do not have a history of safe use (see Figure 1), and their safety must therefore be assessed.

In this context, it is not the number of changes per se that needs to be taken into account, but the pattern of changes and the genotypes and phenotypes resulting from these intended and unintended changes. These basic differences between plant breeding and genetic engineering are overlooked in many publications that try to examine the risks of unintended effects (see, for example, Schnell et al., 2015; Holme et al., 2019).

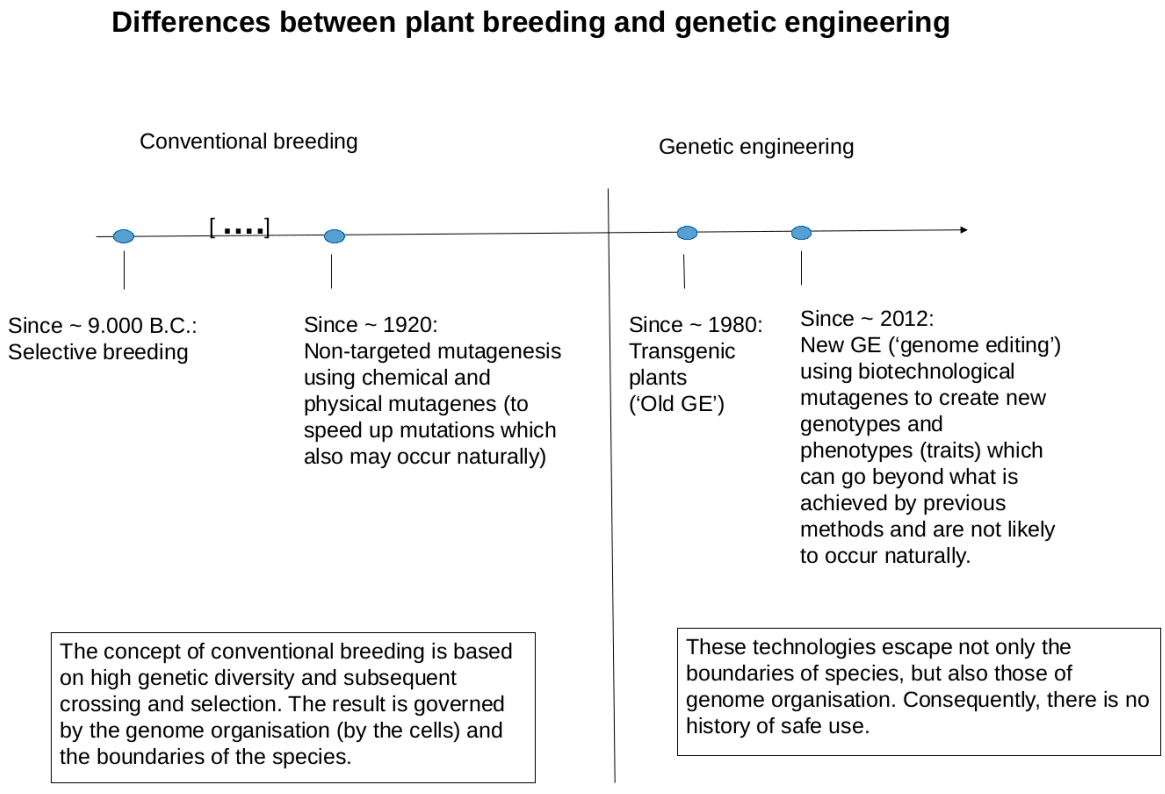

Figure 1: An historical perspective on the differences between plant breedting and genetic engineering.

3. Unintended effects caused by the processes of New GE

The differences between New GE and conventional breeding are relevant to several layers of the discussion about risks of the organisms derived thereof:

There are intended traits, produced without the insertion of additional 'foreign' genes (also called SDN-1 processes), such as changes in oil content (Morineau et al., 2017), protein composition (Sanchez-Leon et al., 2018), sugar concentration (Kannan et al., 2018), plant architecture (Shen et al., 2017), harvesting (Roldan et al., 2017) or biological active plant constituents such as GABA (Nonaka, et al., 2017), which go far beyond what is achieved by conventional breeding (for an overview, also see Kawall, 2021a). These new intended GE traits are the result of specific patterns of genetic changes introduced by 'gene scissors' such as CRISPR/Cas. Just as is the case with transgenic plants, such patterns are unlikely to result from random mutations and conventional breeding methods.

In addition, there are also unintended genetic alterations in the target region (on-target effects) or in other genomic regions (off-target effects) that are specific to gene scissors such as CRISPR/Cas and add a new quality of hazards and risks. For example, larger structural genomic changes such as translocations, deletions, duplications, inversions and scrambling of chromosomal sequences can occur near the SDN target site (as well as at the SDN target site). This has not only been shown to occur in mammalian cells, but also in New GE plants (see e.g., Hahn & Nekrasov 2019).

In brief, there are three aspects of unintended genetic or epigenetic alterations that should be fully addressed together as relevant to risk assessment (see, for example, Kawall et al., 2020; Kawall 2021a; Eckerstorfer et al., 2021; Yang et al., 2022) but are not addressed by several experts (see, for example, Schnell et al., 2015; Holme et al., 2019):

1. Quantity: how many (unintended) alterations are being induced;
2. Quality: this relates to, and depends on, the genomic sites where unintended alterations occur, which genes are unintentionally altered (DNA sequence as well as epigenetic changes), and which regulatory elements and mechanisms might be altered;
3. The type of (unintended) induced alterations: are they point mutations, small insertions or deletions (InDels), larger structural changes such as inversions, translocations, deletions duplications, etc.

Findings of a broad range of unintended effects caused by CRISPR/Cas have already been published. Several publications describe how CRISPR/Cas causes unintended changes, including off-target effects, on-target effects and chromosomal rearrangements (Kosicki et al., 2018; Lalonde et al., 2017; Kapahnke et al., 2016, Haapaniemi et al., 2018; Wolt et al., 2016; Cho et al., 2014; Sharpe & Cooper, 2017; Adikusuma et al., 2018; Kosicki et al., 2020; Biswas et al., 2020; Tuladhar et al., 2019; Ono et al., 2019; Leibowitz et al., 2021; Skryabin et al., 2020; Weisheit et al. 2020; Michno et al., 2020; Norris et al., 2020; Grunewald et al., 2019; Burgio & Teboul, 2020; Liu et al., 2021). These unintended changes can cause a variety of unwanted effects. For example, the integrity of a non-target gene may be compromised if its coding region is cleaved by CRISPR/Cas (e.g. cleavage at off-target-sites). This could lead to changes in the metabolism of the organism that could affect its safety for human health and the environment. Such effects are highly dependent on the genomic context within which such unintended alterations occur (e.g. within a gene, loss of function mutations; outside of genes, unintended alterations in promoters could alter gene expression).

In addition, genome editing is a multi-step process, with inherent and specific risks independent of the purposed traits. For example, in plants, New GE typically makes use of the older genetic engineering ('Old GE') such as non-targeted methods like *Agrobacterium* transformation to deliver the DNA for the nuclease

('gene scissors') into the cells. Thus, in most cases, the result of the first step of the CRISPR/Cas application is a transgenic plant. Only at the end of the multistep process is breeding applied in order to remove the transgenic elements from the plant's genome.

At each stage of the process – including (i) insertion of the DNA of the gene scissors into the cells, (ii) target gene recognition and cutting, and (iii) cellular repair of the genes – specific unintended alterations can occur, with associated risks (for an overview, see Kawall et al., 2020). For example, alterations caused by the non-targeted insertion of transgenic elements in the first step of the process may remain in the plants and impact safety, even if the transgenic elements are removed by further breeding at the end of the process. In this context, there are a number of publications reporting unintended effects arising from the application of 'Old GE' (see, for example, Liu et al., 2019; Gelvin et al., 2017; Forsbach et al., 2003; Jupe et al., 2019; Makarevitch et al., 2003; Windels et al., 2003; Rang et al., 2005).

The following will discuss why many of these unintended changes should be considered as specific to the processes of New GE and understood to go along with a new quality of hazards and risks.

a) Unintended on-target genetic changes

As mentioned, there are patterns of genetic changes that are caused by 'gene scissors' such as CRISPR/Cas that result in the generation of new traits that go beyond what is achieved by previous methods. This is illustrated, for example, in an experiment where wheat is genetically engineered to produce less gluten (Sanchez-Leon et al., 2018). This example is also highly relevant to the discussion about unintended on-target effects: Gluten is suspected to cause inflammations (coeliac disease). It is known that alpha-gliadene peptides contribute to the overall concentration of gluten in bakery products. In the case of this wheat, 35 out of 45 targeted alpha-gliadine genes were altered by CRISPR/Cas (SDN-1) to reduce gluten in food products.

This may appear to be a successful and precise application of the gene scissors, however, the changes lack sufficient predictability: There are many different types of insertions and/or deletions that are specific to each of the targeted genes. Also, in some cases, additional DNA was inserted into the target site (see Figure 2). Consequently, the European Food Safety Authority came to the conclusion that, in this case, the intended and unintended changes at the target sites pose new challenges for risk assessment: *"(…) the large number of mutations required to achieve gluten-free wheat is far beyond any plant previously assessed. This is likely to require SynBio approaches to correctly identify all gliadins and glutenins in the hexaploid genome of bread wheat and to identify an engineering strategy that introduced mutations of the correct nature and positions in each gene to prevent the accumulation of any peptide fragments associated with initiation of the inflammatory cascade"* (EFSA, 2021).

This case shows that, even where changes are 'successfully' introduced in the target genes, complex questions in regards to the safety of these plants need to be considered: Each and every targeted genetic site needs to undergo a detailed examination to determine if the alpha-gliadine proteins are still produced, or if new proteins are produced unintentionally, or if any other unintended effects may occur. It is clear that such unintended variations of genetic changes caused by New GE go along with a new quality in hazards and risks, even if no off-target genetic changes are identified.

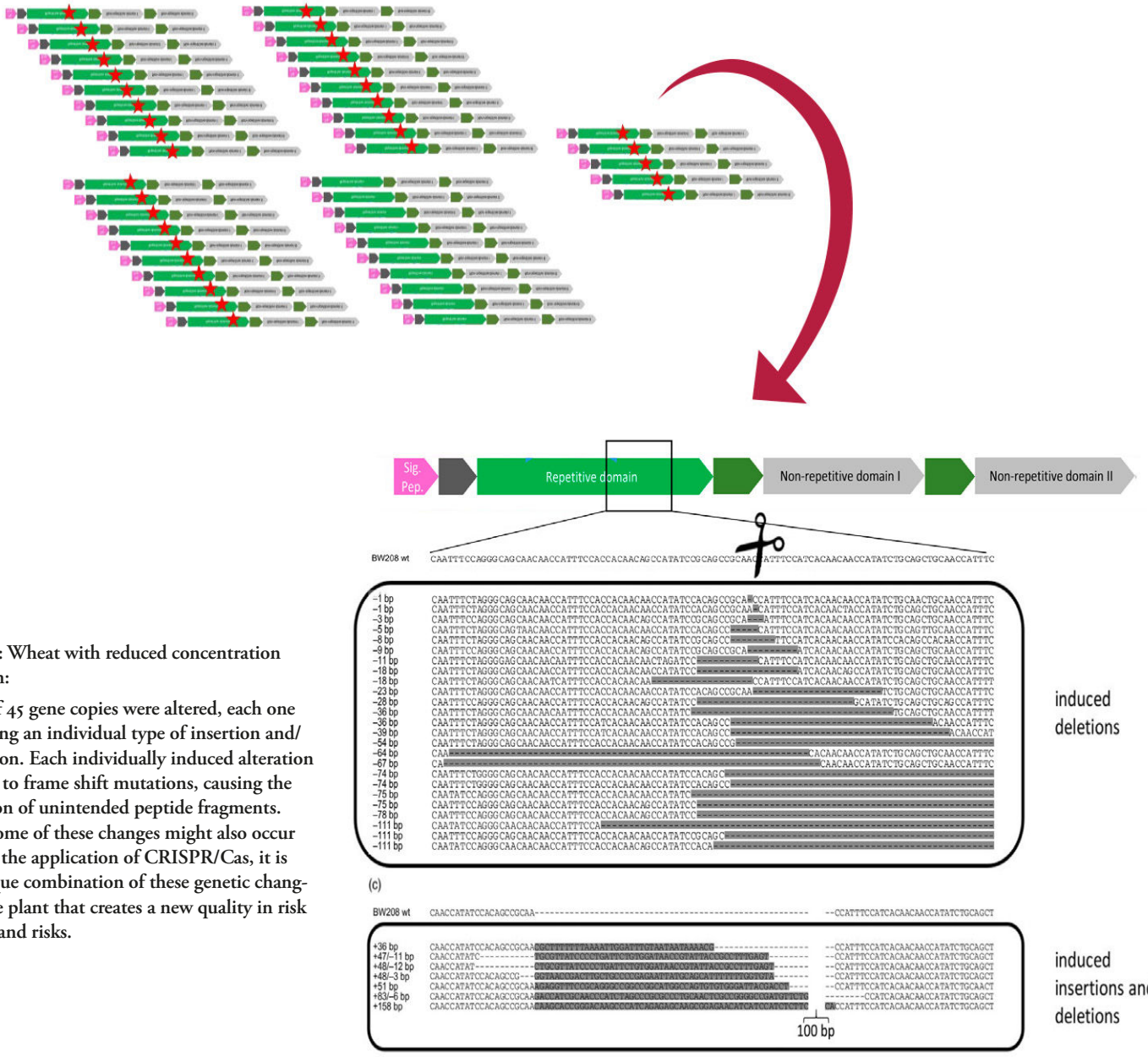

Figure 2: Wheat with reduced concentration in gluten:

35 out of 45 gene copies were altered, each one containing an individual type of insertion and/or deletion. Each individually induced alteration can lead to frame shift mutations, causing the formation of unintended peptide fragments. While some of these changes might also occur without the application of CRISPR/Cas, it is the unique combination of these genetic changes in one plant that creates a new quality in risk hazards and risks.

In addition, specific unintended on-target effects often include integration of DNA from vector DNA derived from transformation processes, where, for example, bacterial DNA was unexpectedly integrated (e.g. Li et al., 2015; Andersson et al., 2017; Zhang et al., 2018). In animal cells, it was found that unintentional inserted foreign DNA fragments may not only be derived from the vector construct (Norris et al. 2020), but may also be derived from the genome of the bacteria that is used to multiply the vector DNA (e.g. Escherichia coli) or, surprisingly, taken up from the source of the growth medium, such as bovine or goat DNA, or retrotransposon (Ono et al., 2015, 2019). Overall, the CRISPR/Cas9 system has been confirmed to have a high frequency of integration into the target site, resulting in large deletions in target sites (Lee et al., 2019; Yang et al., 2022).

b) Unintended off-target genetic changes

The CRISPR/Cas machinery is particularly known for its potential to confuse target regions with specific off-target regions, in addition to causing unintended insertion of additional genes, decoupling of genes and other specific genomic alterations (of categories such as inversions, deletions or rearrangements) that are unlikely to emerge from spontaneous mutations or physical and chemical mutagenesis (see, for example, Biswas et al., 2020; Braatz et al., 2017; Höijer et al., 2022; Kawall et al., 2020). In some cases, unusual patterns of inheritance have also been observed, thus escaping the Mendelian rules (Höijer et al., 2022; Yang, et al., 2022).

As a result, similar to the case with on-target genetic changes, off-target effects can also cause patterns of genetic changes that go beyond what can be achieved by conventional breeding, resulting in specific and hazards and risks of a new quality. Yang et al. (2022) give some overview of irregular genetic changes and specific unintended effects caused by intrinsic factors of the CRISPR/Cas9 systems in plants. These include off-target DNA cleavage, repetitive unit deletion, and indels of various sizes (Zhang et al. 2014; Chakrabarti et al., 2019; Manghwar et al. 2020; Molla and Yang 2020; Kapusi et al., 2017). In this context, for example, the dosage of CRISPR/Cas9 complexes in cells expressed can result in a significant increase in off-target mutation frequency (Ordon et al., 2017; Zhang et al., 2018).

Some of these unintended effects (on-target and off-target) are summarized in Figure 3.

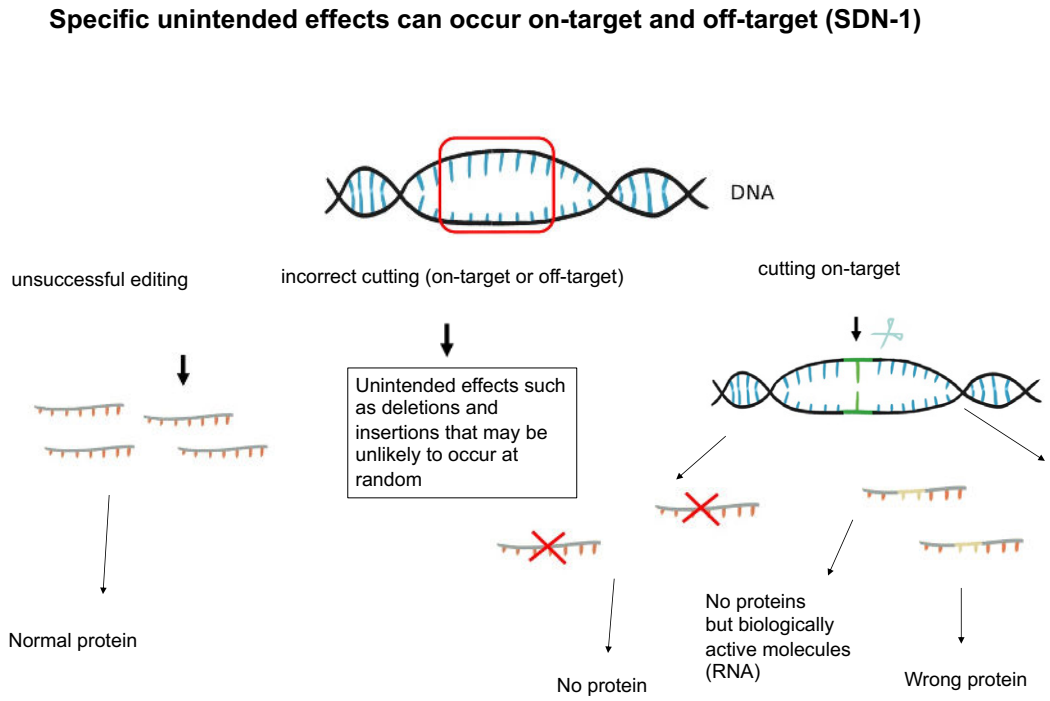

Figure 3: Overview of some types of unintended on-target and off-target changes caused by SDN-1 processes that can result in specific effects and create new quality in hazards and risks.

c) Unintended metabolic and physiological effects

The mechanisms and findings presented above are mostly related to changes at the level of DNA and potential gene products emerging from the specific genomic sites. Additionally, unintended metabolic and physiological effects can occur that are relevant to the interaction of New GE organisms with the environment.

As Table 1 shows, there are unintended effects observed in numerous species, both plants and animals. The unintended metabolic and physiological effects may be caused by intended or unintended genetic changes as discussed above. Some of these effects are known from conventional breeding (such as costs of MLO resistance), however, the genotypes and effects (intended or unintended) caused by New GE go beyond what is achieved by previous methods (see Kawall, 2021a).

Table 1: Select examples of unintended metabolic and physiological effects observed in plants and animals genetically engineered with CRISPR/Cas, with hypothesized risks.

Species	Intended trait	Unintended metabolic and physiological effects and hypothesized risks
Wheat	Mildew resistance (MLO)	Growth aberration, accelerated senescence, induced necrosis, increased susceptibility to other fungal pathogens. (Spanu, 2022)
Wheat	Decreased acrylamide content	Reduced growth and germination rate, potentially increased susceptibility to fungal plant pathogens. (Raffan et al., 2021)
Camelina	Altered oil quality	Weakened defense mechanisms against biotic (pathogens) or abiotic (climate change) stressors. (Kawall 2021b)
Tomato	Enhanced GABA content	The changes in plant composition may also cause unintended health effects at the stage of consumption. Furthermore, unexpected reactions of the plants to environmental stress conditions are not unlikely. (Nonaka et al., 2017)
Tomato	Accelerated domestication	Differences in plant composition are observed in comparison to previously bred tomatoes. These differences may also impact health at the stage of consumption. (Zsögön et al., 2018)
Rice	Improved salinity tolerance	Enhanced invasiveness might occur in weedy rice after hybridisation. (Zhang et al., 2018)
Sea bream	Faster weight gain	Skeletal disorders (such as abnormal position of vertebra), which are not unlikely to impact animal welfare. (Kishimoto et al., 2018)

Unintended effects such as those listed in Table 1 could have serious adverse impacts on the environment, plant health, agricultural yield, pesticide use, animal welfare and/or food safety. If grown in fields, the interactions between New GE organisms and the environment, including pests, pathogens, climatic conditions, etc. adds further complexity and risk.

These unintended effects are the result of interactions in the complex networks of genes, proteins and other biological active molecules. Such unintended metabolic and physiological effects can also emerge in cases when genetic intervention is targeted and precise.

4. Systemic risks

Because techniques such as CRISPR/Cas escape the mechanisms of natural genome organisation, the resulting organisms need to be considered as being different to those plants and animals derived from conventional breeding and physical or chemical mutagenesis. As explained above, both intended and unintended effects from the processes of New GE can result in genotypes and phenotypes which, in comparison to conventionally bred plants, are as unlikely to occur as those resulting from the insertion of 'foreign' genes for the production of Bt toxins, for example.

Therefore, New GE plants cannot be equated with plants adapted by evolutionary processes and cannot be regulated like all other food plants, based on the intended new characteristic(s). Instead, the processes by which new characteristics are introduced into organisms need to be taken into account, to examine all intended and unintended effects and their possible related hazards and risks (Kawall et al., 2020).

More generally, traditional breeding cannot largely or wholly be replaced by New GE because the use of these genomic techniques will be accompanied by so many risks, uncertainties and unknowns. The release of genetically engineered organisms cannot be regarded as being neutral because of the risks posed to biodiversity, animal welfare, conventional and organic agriculture, traditional food production systems, and human health and food safety. Plants that have not adapted via evolutionary processes can disturb or interrupt ecological networks in many ways.

Decision making over the use of genetic engineering and the introduction of plants and animals derived from Old or New GE should, therefore, be guided by the precautionary principle, to prevent ecosystems and food systems from being flooded with too many risks, uncertainties and unknowns within a short period of time (and expanding over time). The potential to scale uncertainty and harm is a specific feature of New GE where it is possible to make biological changes at large geographical scales and across many species (Heinemann et al., 2021).

As with the need to reduce the use of plastics and toxics such as pesticides, there is a need to restrict the introduction of organisms with human-made genetic design into the environment. Environmental problems created by the release of GE organisms may last as long as, or longer than, those from plastics and pesticides, with impacts on many future generations.

Consequently, not only the risks associated with individual GE organisms, but also the systemic risks and potentially disruptive effects of using New GE need to be taken into account. Therefore, a comprehensive and prospective technology assessment needs to be conducted to address systemic and long-term risks. This is especially relevant if, within a short period of time, many of these genetically engineered organisms are introduced into the environment, agro-ecosystems and food systems.

5. Conclusion: The need for precautionary regulation

There is increasing evidence that the intrinsic factors of the New GE techniques deserve much more attention from regulators. For example, according to Yang et al. (2022), *"mutation locations and scales, potential off-target modifications, complexity of the introduced changes, and novelty of the developed traits"* make it necessary to apply *"rigorous research on genome-editing applications and reliable techniques for risk assessments of genome-edited plants".*

Kawall (2021a), in investigating the generic risks that go along with the application of the CRISPR/Cas machinery, concludes, *"In summary, this review here shows that about half of the market-oriented plants developed by SDN-1 applications contain complex alterations in their genome (i.e., altering multiple gene variants or using multiplexing). It also illustrates that data on both the process- and the end-product are needed for a case-by-case risk assessment of genome edited plants. The broad range of genetic alterations and their corresponding traits reflects how diverse and complex the requirements are for such a risk assessment."*

Eckerstorfer et al. (2021) come to a similar conclusion: *"To this end, we suggest that two sets of considerations are considered: (1) trait related-considerations to assess the effects associated with the newly developed trait(s); and (2) method-related considerations to assess unintended changes associated with the intended trait(s) or with other modifications in the GE plant. (…) Based on these considerations, further guidance should be developed to ensure the high safety standards provided by the current regulatory framework for GMOs in the EU for GE plants in an adequate and efficient way, taking into account the existing knowledge and experience in a case-specific manner. This guidance should thus strengthen the case-specific approach that is recommended by numerous EU and Member States institutions."*

The unintended effects that can result from the use of New GE techniques cannot be overlooked without jeopardizing environmental and food safety. Instead, all New GE organisms need to be subject to mandatory, independent government risk assessment before release into the environment or market.

Furthermore, the use of New GE technology in agriculture requires comprehensive and prospective technology assessment to address systemic risks. In accordance with the precautionary principle, such technology assessment should also rely on in-depth consideration of the need for the technology and the alternatives that could be made available. This technology assessment should be conducted with the participation of the public and affected communities, for example in consultation with farmers.

Without precautionary regulation of new GE:

- large numbers of genetically engineered organisms can be expected to be released in an uncontrolled way within a short period of time;
- the risks of serious damage to biological diversity, ecosystems and agricultural systems will increase;
- access to data needed for risk assessment by independent experts would not be available;
- no information would be available to track and trace the New GE organisms and food products derived from them;
- human health effects may be introduced and could accumulate unnoticed in the food system;
- few measures would be available to mitigate the uncontrolled spread of these organisms in the environment;
- organic and other GE-free food and farming could no longer be protected from GE contamination.

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