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New Genomic Techniques and unintended genetic changes: EFSA ‘overlooked’ most of the relevant publications

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Summary

In a document, recently published (EFSA, 2022a), EFSA has created the impression that there is, in most cases, no need to take the unintended genetic changes caused by NGT processes into account. EFSA appears to assume that the unintended genetic changes and the associated risks could not be distinguished from those resulting from conventional breeding. Consequently, the approach as suggested by EFSA would mean a substantial reduction in current standards of risk assessment.

It looks like the EFSA assumptions largely originate from inadequate data: in the context of its previous opinions, the authority has stated several times that it did not have a mandate to comprehensively assess all relevant scientific publications. On the contrary, it seems that EFSA has, in fact, simply ‘overlooked’ most of the relevant publications.

Indeed, many publications show that the multi-step processes of NGTs are associated with intended and unintended genetic changes that can be different to those resulting from conventional breeding methods. This is especially relevant for organisms engineered with ‘gene scissors’ such as CRISPR/Cas. Such differences between conventional breeding and NGTs can be easily overlooked, but can, nevertheless, have serious consequences: if overlooked, hazardous genetic changes can rapidly spread throughout large populations.

Unintended genetic changes caused by NGT processes that are unlikely to result from conventional breeding are highly relevant to the risk assessment of NGT organisms. The reason: these changes may trigger risks that were not anticipated and which may go beyond what is known from conventional breeding.

As shown in this backgrounder, EFSA only mentions around 20 percent of the relevant publications forwarded by Testbiotech during the public consultation process. Similarly, several publications provided by experts from EU Member States were not taken into account. Furthermore, even if publications were listed in the EFSA references, none of these were addressed in the reports in order to systematically examine unintended effects caused by NGT procedures.

Consequently, the criteria proposed by EFSA for the risk assessment of NGT plants are not sufficiently science-based. Testbiotech is, therefore, demanding that EFSA withdraws its proposal (‘statement’) for the future risk assessment of plants derived from new genome techniques (NGT).

1. Introduction

Current EU regulation requires that genetically engineered plants, including those derived from new genomic techniques (NGT), are risk assessed for all intended and unintended genetic changes. This includes risks to health and the environment. The regulations require that direct and indirect effects, which may be immediate, delayed or cumulative, are taken into account. However, according to EFSA, future risk assessment would mostly only take the intended biological characteristics of the plants into account and not the unintended genetic changes resulting from NGT processes.

The European Food Safety Authority (EFSA) published a ‘statement’ in October 2022 on the future risk assessment of plants derived from new genome techniques (NGT or New GE). The statement included a proposal to considerably reduce current regulations for genetically engineered plants (EFSA, 2022a). This would mean, in most cases, that future risk assessment would only take the intended characteristics of the plants into account and disregard any unintended genetic changes caused by the genetic engineering processes. As a result, many NGT plants could be brought to market without undergoing detailed risk assessment.

2. Unintended genetic changes caused by NGTs are relevant to risk assessment

Many publications show that the multi-step processes of NGTs are associated with unintended genetic changes that can be different to those resulting from conventional breeding methods. This is especially relevant for plants engineered with ‘gene scissors’ such as CRISPR/Cas. Such differences between conventional breeding and NGTs can be easily overlooked, but can, nevertheless, have serious consequences: if hazardous, unintended genetic changes go unnoticed, they can rapidly spread throughout large populations.

The processes involved in NGTs may cause unintended genetic changes which are different to those which can be expected from conventional breeding (including random mutagenesis):
(1) Gene scissors make the genome available for change to a much greater extent compared to conventional breeding methods. The likelihood of unintended genetic changes occurring at specific genomic sites is, therefore, higher with NGTs.
(2) The sites of unintended genetic changes and the resulting combinations (of intended and unintended genetic alterations) caused by NGTs can be vastly different compared to those resulting from conventional breeding.
(3) NGTs are based on a complex multistep process including old methods of genetic engineering to introduce the DNA of the gene scissors into the cells. This first step, which is most often applied in plants, can also cause unintended genetic changes that are different to those resulting from conventional breeding.

Unintended genetic changes caused by NGT processes that are unlikely to result from conventional breeding (including random mutagenesis) are highly relevant to the risk assessment of NGTs. The reason: these changes may trigger adverse effects (direct or indirect, immediate, delayed or accumulated) that were not anticipated and which may go beyond what is known from conventional breeding. The following three categories contain more detailed explanations of these issues.[1]

2.1 The potential for the occurrence of unintended genetic changes that are generic to NGTs

The category below includes publications showing that ‘gene scissors’ mechanisms make the genome available to a greater extent compared to conventional breeding (including random mutagenesis). This is closely associated with a specific potential to trigger unintended (and intended) genetic changes.

NGTs can be used to achieve genomic changes that reach beyond what is known from conventional breeding, even without the insertion of additional genes. Compared to conventional breeding methods (including random mutagenesis), NGTs can overcome the boundaries of natural genome organization that have evolved naturally from evolutionary processes. Relevant factors in this respect include repair mechanisms, gene duplications, genetic linkages and other epigenetic mechanisms (see, e.g. Belfield et al., 2018; Filler Hayut et al., 2017; Frigola et al., 2017; Halstead et al., 2020; Huang & Li, 2018; Jones et al., 2017; Lin et al., 2014; Monroe et al., 2022; Wendel et al., 2016), thus making the genome much more extensively available for genetic change (Kawall, 2019; Kawall et al., 2020). The resulting genotypes (the patterns of intended and unintended genetic changes) can be vastly different compared to those derived from conventional breeding, both in regard to intended and unintended changes (Kawall, 2021a; Kawall, 2021b).

2.2 Findings on unintended genetic changes that are specific to NGTs

The following category includes publications indicating that many unintended on-target and off-target genetic changes caused by NGTs are unlikely to result from other processes.

In a similar way to intended traits, unintended effects can also cause patterns of genetic change that go beyond what can be achieved with conventional breeding, and thus lead to specific risks. Unintended genetic changes include off-target DNA cleavage, repetitive unit deletion, indels of various sizes, larger structural changes in the targeted genomic region and the unintended insertion

1 The following passages are taken in part from Testbiotech (2022b).

of transgenes. While some of these ‘types’ of genetic alteration might also be observed in conventional breeding (EFSA, 2022b), the probability of these changes occurring at a specific site in the genome and the resulting genotype can be very different (for overview see Kawall, 2021b). If these unintended effects are overlooked, they may spread rapidly throughout large populations. Moreover, if the seeds are used for further propagation and breeding, potentially hazardous genetic alterations can remain undetected for a longer period of time and may also accumulate.

Findings on 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 (Adikusuma et al., 2018; Biswas et al., 2020; Burgio et al., 2020; Cho et al., 2014; Grunewald et al., 2019; Haapaniemi et al., 2018; Kapahnke et al., 2016, Kosicki et al., 2018; Kosicki et al., 2020; Lalonde et al., 2017; Leibowitz et al., 2020; Liu et al., 2021; Michno et al., 2020; Ono et al., 2019; Sharpe, 2017; Skryabin et al., 2020; Tuladhar et al., 2019; Weisheit et al., 2020; Wolt et al., 2016).

Unintended genetic alterations in the target region (on-target) or in other genomic regions (off-target) specific to gene scissors, such as CRISPR/Cas, have been described several times. 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 actual SDN target site) which would otherwise be unlikely to occur (see e.g., Hahn & Nekrasov, 2019). In addition, specific unintended on-target effects often include the integration of DNA from vector DNA derived from transformation processes, where, for example, bacterial DNA was unexpectedly integrated (e.g. Andersson et al., 2017; Li et al., 2015; Zhang et al., 2018). Overall, the CRISPR/Cas9 system has been confirmed to have a high frequency of integration into the target site, resulting in large deletions at the target sites (Lee et al., 2019; Yang et al., 2022).

In general, the CRISPR/Cas machinery is known for its potential to confuse target regions with specific off-target regions. This is in addition to causing the 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 happen with spontaneous mutations or physical and chemical mutagenesis (see, for example, Biswas et al., 2020; Braatz et al., 2017; Hahn & Nekrasov 2019). In some cases, unusual patterns of inheritance have also been observed, which as such escape the Mendelian rules (Yang et al., 2022).

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).

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

2.3 Unintended genetic changes caused by the process of introducing the gene scissors into the cells

This category includes publications showing that unintended genetic changes (that are unlikely to result from processes used in conventional breeding) are also caused by the insertion of the DNA necessary for production of the gene scissors into the genome of the plants.

New GE is a multi-step process associated with inherent and specific risks that can occur independently of the desired traits. For example, NGTs such as CRISPR/Cas applications in plants typically use older genetic engineering (‘Old GE’) methods, i.e. non-targeted methods to deliver the DNA coding for the nuclease into the cells. Thus, in most cases, the outcome of the first step of a CRISPR/Cas application is a transgenic plant which may show a broad range of unintended genetic changes that are unlikely to emerge from conventional breeding. Conventional breeding is only used at the end of the multistep process to remove the transgenic elements from the plant genome (segregation breeding). However, without adequate standards of risk assessment in place, the unintended genetic changes may remain undetected in the genome, which could then spread rapidly and widely throughout populations and also accumulate after crossings with other NGT plants.

The mechanisms and outcomes of these technical processes for the insertion of genes, such as biolistic methods and usage of *Agrobacterium tumefaciens*, cannot be equated to effects occurring naturally or used in previous breeding methods. For example, Yue et al. (2022) identified larger and smaller insertions as well as deletions caused by the biolistic method of gene insertion into papaya. A larger insertion consisted of 77 rearranged and translocated fragments; a larger deletion included 44 genes. More than 600 genes were changed in their activity. The changes caused by the method of genetic engineering could be clearly distinguished from other genomic changes, which have evolved over (around) 4000 years of domestication of papayas. In conclusion, the processes used for the technical insertion of DNA can cause effects which are different in their scale, in the sites and in the patterns of genetic change as well as in their biological characteristics when compared to those resulting from non-regulated breeding methods or natural processes. This is also true even if no additional genetic information is added to the gene pool of a species. Such effects may be related to epigenetic regulation, the disruption of genes, position effects, open reading frames, the unintended introduction of additional genes, changes in gene expression and genomic interactions which can involve plant constituents, plant composition and agronomic characteristics (Forsbach et al., 2003; Gelvin et al., 2017; Jupe et al., 2019; Liu et al., 2019; Makarevitch et al., 2003; Rang et al., 2005; Windels et al., 2003; Yue et al., 2022).

3. EFSA ‘overlooked’ around 80 % of the relevant publications

In several public consultations, Testbiotech and other experts listed a large number of publications related to the three categories described above. Testbiotech subsequently compared the publications introduced during the consultations[2] to those included in EFSA reference lists (EFSA, 2020a; EFSA, 2021; EFSA, 2022a; EFSA, 2022b; EFSA, 2022c; JRC, 2021). The result is astonishing: EFSA excluded around 80 % of the relevant publications from its reports (see figure 1). As Annex 1 shows, Testbiotech forwarded (at least) 44 relevant publications, but EFSA only mentions 10 of them. Similarly, several publications listed by experts from Member States were not taken into account. Furthermore, even if publications were listed in the EFSA references, none of these were addressed in the reports in order to systematically examine unintended effects caused by NGT procedures.

2 2021a, 2021b, 2022a; 2022b; Testbiotech & CBAN, 2022.

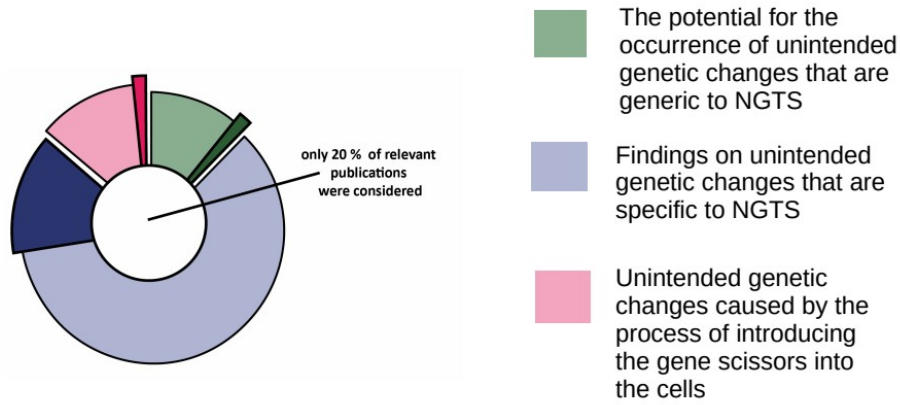

Figure 1: Percentage of relevant publications taken into account by EFSA or JRC (EFSA, 2020a; EFSA, 2021; EFSA, 2022a; EFSA, 2022b; EFSA, 2022c; JRC, 2021) in the three categories listed.

EFSA failed to raise several crucial questions which, therefore, went unanswered, e.g. which requirements are necessary and which methods are suitable for detecting and assessing:

- specific unintended genetic and epigenetic alterations caused by the processes of NGT?
- specific unintended effects caused by the NGT traits in the organisms?
- direct and indirect, intended and unintended effects caused by the NGT organisms in the ecosystems?
- specific intended and unintended effects caused by the processes of NGT relevant to food & feed safety?

There may be several explanations for these findings. For example, EFSA may believe that not all publications are relevant because some investigated other organisms rather than plants. However, unless EFSA actually assesses the publications, no such statement or ‘excuse’ is acceptable. For example, several examinations of animal (human) cells would need to be repeated in plants to find out how relevant the findings are to NGT plants. So far, the causes and risks of unintended effects caused by NGT processes in plants have not been subject to comprehensive investigation. Therefore, it is important to also identify research gaps by taking into account publications from other organisms.

4. The EFSA mandate

There is a general problem with the role and mandate of the European Food Safety Authority (EFSA). EFSA was requested by the EU Commission to investigate potential new hazards and risks that various NGTs could pose in comparison to conventional breeding or established genomic techniques. The aim was to investigate whether the current guidelines for the risk assessment of

transgenic plants are sufficient for NGT plants.[3] However, the wording of mandate appears to have linked two different questions that should have kept separated:

- On the one hand, the applicability of the current guidelines does not per se answer the question of whether and how the NGTs should be regulated in future. At the same time, observations made by the Commission on the EFSA reports give the impression that such conclusions could be drawn.
- On the other hand, the question to which extent the NGT processes can cause unintended genetic changes unlikely to occur from conventional breeding (or from any other non-regulated process for plant breeding) may be decisive as far as regulation of NGTs is concerned. However, the Commission does not explicitly address this question. Consequently, it was neither systematically investigated nor answered by EFSA in a way that could satisfy the necessary scientific standards.

Prior to any further steps being taken to discuss potential hazards, EFSA should have first of all have provided a comprehensive literature review of the generic potential of NGTs to cause specific unintended genetic changes unlikely to occur with conventional breeding methods. This is also stipulated in the provisions of the EU regulations (EU Directive 2001/18/EC and Commission Directive (EU) 2018/350), which require the assessment of any unintended genetic changes caused by the NGT processes unlikely to result from conventional breeding. The reason: such changes may trigger adverse effects (direct or indirect, immediate, delayed or accumulated) that were not anticipated and which may go beyond what is known from conventional breeding, and thus make it necessary to assess any unintended genetic changes independently of any comparison with other regulated techniques. In addition, the unintended effects caused by 'old GE' techniques also have to be taken into account insofar as they are used to introduce the DNA required for the gene scissors into the genome of plant cells.

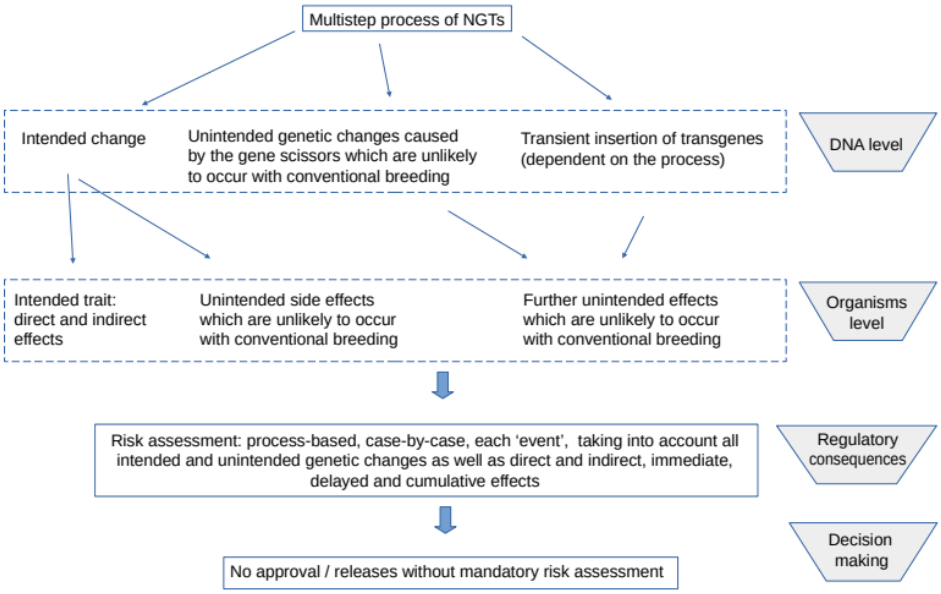

Figure 2: Overview of the steps in the risk assessment of NGTs required by law (source: Testbiotech 2022b)

3 EU Commission references: Ares(2021)3837829, Ares (2019)2499590, Ares(2018)3356496, Ares(2012)573179 and Ares(2011)201516

Despite these scientific and legal requirements, EFSA (2020a and 2022d) explicitly states that their experts did not conduct a full literature search to compile a reasoned overview of unintended genetic changes, as this was not within their mandate. For example, in the response to the most recent consultation (EFSA 2022d) EFSA states: "*Moreover, the GMO Panel was not mandated to provide a comprehensive literature review on the SDN-based technology*[4] *and its unintended effects.*"

As a result, the reports and statements published by EFSA reveal serious gaps in data collection and data assessment. As Annex 1 shows, the number of relevant publications waiting for the attention of EFSA has increased substantially in recent years. Without taking these (or any other relevant publications) into account, no conclusion can be drawn on the requirements for the regulation of NGT organisms.

5. Conclusions

EFSA has created the impression that there is, in most cases, no need to take the unintended genetic changes caused by NGT processes into account. EFSA appears to assume that the unintended genetic changes and the associated risks could not be distinguished from those resulting from conventional breeding. Consequently, the approach as suggested by EFSA would mean a substantial reduction in current standards of risk assessment.

The EFSA assumptions appear to originate from inadequate data: in the context of its previous opinions, the authority has stated several times that it did not have a mandate to comprehensively assess all relevant scientific publications. On the contrary, it seems that EFSA has, in fact, simply 'overlooked' most of the relevant publications.

As a result, the criteria proposed by EFSA for the risk assessment of NGT plants are not sufficiently backed by science. Testbiotech is, therefore, demanding that EFSA withdraws its proposal ('statement') on the future risk assessment of plants derived from new genome techniques (NGT) (EFSA 2022a).

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4 SDN means 'site directed nucleases' which is another, more technical expression for 'gene scissors'.

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Annex: Overview of relevant publications forwarded by Testbiotech to EFSA during the consultation process, grouped into three categories

1. The potential for the occurrence of unintended genetic changes that are generic to NGTs (* mentioned by EFSA or JRC).

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