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SITE-DIRECTED MUTAGENESIS – A CHANCE TO MEET ENVIRONMENTAL CHALLENGES AND PROVIDE HEALTHY FOOD FOR PEOPLE OR AN UNACCEPTABLE HAZARD TO HUMANS, ANIMALS, AND THE ENVIRONMENT. CONSEQUENCES OF THE EUROPEAN COURT OF JUSTICE JUDGMENT IN CASE C-528/16

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ABSTRACT

One of the EU's strategic goals is to reduce the environmental and climate footprint of the EU food system and strengthen its resilience, while ensuring food security for European citizens. Thus, the EU Farm to Fork strategy, which is one of the central pillars of the European Green Deal, set ambitious targets for 2030 to reduce pesticide use in agriculture by 50% and fertilizers use by 20%, with a concomitant 50% reduction of nutrient leakage to surface and groundwater. Additionally, it is recommended that at least 25% of the EU agricultural land shall be kept under organic farming. These goals are far-reaching, but several recent studies indicate that implementing them without significant progress in research and innovation (R & I) may result in a yield decrease by up to 30%, depending on the crop, and an increase in the price of agricultural commodities by up to 18%. Especially affected would be horticulture due to its high dependence on plant protection against pests and diseases. Therefore, the studies recommend accelerating plant breeding in order to produce new plant cultivars genetically resistant to pests and diseases and better equipped to cope with abiotic stresses like limited nutrition and water deficit. The progress in classical plant breeding is a lengthy process. It is especially slow in the case of woody species, like most fruit plants, due to their long juvenile periods and limited genetic variance. Recent advances in functional genomics, bioinformatics, and molecular methods provided tools that speed up the breeding process significantly. Several site-directed mutation technologies allow modifying a specific gene at a predefined site, by deletion or insertion of single or multiple nucleotides, without affecting off-target genes. Several valuable cultivars have been bred so far using these methods, and a large number of others are under trials. However, their release will be severely impeded by the decision of the Court of Justice of the European Union, dated 25 July 2018, that the release of organisms obtained by site-specific mutations, as opposed to organisms obtained by induced random mutation, is controlled by Directive 2001/18/EC2 on genetically modified organisms. This paper reviews the new generation breeding techniques, especially site-directed mutagenesis, and their benefits as well as potential hazards to consumers and the environment.

Key words: Court of Justice of the EU, case C-528/16, EU Green Deal Policy, healthy food, new genomic techniques, site-directed mutagenesis

INTRODUCTION

Facing the alarming increase of agriculture-borne environmental pollution and biodiversity decline on agricultural lands, the European Commission launched in 2020 the Green Deal initiative, which includes two key strategies: Farm to Fork Strategy and Biodiversity Strategy for 2030. Their aims are to reduce by 2030 the use of chemical pesticides by 50% and the use of fertilizers by 20% with a concomitant 50% reduction of nutrient losses from the fields due to their leaching to surface waters and emission of ammonia/nitrous oxides to the atmosphere (EC 2020a). To mitigate biodiversity loss, it is proposed to set aside at least 10% of agricultural

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lands for establishing high-diversity landscapes and to transform at least 30% of Europe's lands and seas into effectively managed protected areas, with 10% of them strictly protected. It is also expected that by 2030 at least 25% of cultivated lands will be under organic farming (EC 2020b).

Since the publication of these strategies, various studies have been undertaken to analyze their impacts on agriculture and food security in the European Union (EU). The most notable are extensive analyses made by the teams from European Joint Research Centre (Barreiro-Hurle et al. 2021), Wageningen University (Bremmer et al. 2021), Kiel University (Henning et al. 2021) and USDA (Beckman et al. 2020). Despite various methodologies applied and various scenarios studied, the overall conclusion from all these studies is that implementing the strategies will produce benefits in terms of climate and biodiversity, but with the currently available agricultural technologies and crop plants germplasm, it would come at a price. The average yield would decrease by 12%, but for fruit crops, like apples, it would be as much as 30%. The prices for fruits and vegetables would increase by 15%. Despite higher prices for agricultural commodities, farm revenues will decrease from 9% to 15% due to lower yields and more costly crop management. The full implementation of both strategies would also affect strongly the EU trade balance of agricultural commodities. In the case of fruits and vegetables, the current net import of 10 mln tonnes would increase to 22 mln tonnes. The estimated loss of EU gross domestic products (GDP) ranges from EUR 30 bln according to the Wageningen study to USD 70 bln according to USDA.

It shall be also noted that the reduction of pesticides use can result in quality problems, especially the accumulation of mycotoxins in some agricultural commodities, which would make them unsuitable as food or feed. besides affecting yields, In addition to affecting yields, reducing the consumption of nutrients will also have an impact on the sensory quality, storability, and shelf life of fruit and vegetables, which will have a further price impact. This would need further fundamental and applied research.

To help mitigate the expected negative impacts of implementing From Farm to Fork and Biodiversity strategies, especially regarding the reduction of pesticides and nutrients use, it is recommended to accelerate research on innovative crop protection techniques, such as biocontrol, biostimulants, precision agriculture, mechanical and other alternative weed control methods, and on plant breeding. In order to shorten the breeding process, removing legislative barriers to new breeding techniques (NBTs) and new genomic techniques (NGTs) shall be considered. It is especially important in the case of woody perennials, such as most fruit plant species, for which classical breeding is very lengthy due to their long juvenile period.

In this paper, the advantages of site-directed mutagenesis and their potential usefulness in mitigating the negative impacts of implementing European strategies From Farm to Fork and Biodiversity Strategy 2030 are presented.

Mutations in plant breeding

Mutations play a major role in plant breeding, including fruit plants. Spontaneous hereditary changes in tree shape, growth vigor, fruit size, color and taste were observed by orchardists since times immemorial. Some of these variants showing desired phenotypes, like improved fruit quality or tree growth were selected and propagated clonally by grafting or rooted cuttings and eventually were established as new varieties. According to Kharkwal (2012), the mutant crops were already reported in China as early as 300 BC. The first written report on spontaneous variations in plants in European literature comes from Charles Darwin's book "Variation of Animals and Plants Under Domestication", where he described several mutations in fruit trees and noted that nectarine is a hairless mutant of a peach (Darwin 1868). However, it was Hugo de Vries (1906) who coined the term "mutation" and postulated that it is a driving force of variability. Later on, many variants of original varieties having altered phenotypes have been described (Granhall 1954). Spontaneous mutations can occur due to errors in DNA replication, endogenous DNA lesions, or polyploidy, but they are rare. It is estimated that no more than 8–10 base pairs mutate spontaneously per generation in eukaryotic genomes (Drake et al. 1998), but they are rarely expressed phenotypically ("silent mutations") and their molecular basis is rarely identified.

Nevertheless, a large number of commercially important fruit cultivars grown today have originated that way (Janick 2011), and even now spontaneous mutants are still being selected by fruit growers and breeders and registered as new cultivars. The spontaneous mutations usually affect single traits, like fruit color or size, but also could affect important plant developmental processes, like inhibition of fruit ripening in tomato mutants SPL-CNR, NAC-NOR, and MADS-RIN (Adaskaveg et al. 2021) or inhibition of lateral branching in apple mutant cultivar 'Wijcik' (Wolters et al. 2013).

Induced mutations

Induced mutations provided breeders with the mechanism for controlling mutational breeding. It has been introduced in the 1920s when it has been discovered that heritable mutations could be induced in plants by means of irradiation or chemical treatments (Stadler 1928).

Mutation can be induced by treatment with ionizing radiation (X-rays, gamma-rays, neutrons, beta particles, alfa particles, ion beams and ultraviolet – UV light) or chemical mutagens (alkylating agents, azides, hydroxylamine, some antibiotics, and analogs of nucleobases). The mutation caused by ionizing radiation and chemical mutagens can affect both the nuclear genome and the genome of plastids (chloroplasts) and mitochondria (Pathirana 2011; Fluhr et al. 1985).

The first experiments with mutation breeding were done using X-rays generated in the Roentgen apparatus, which was later replaced by a more convenient treatment with gamma rays emitted by radioactive cobalt (^{60}Co) (Beyaz & Yildiz 2017). The most effective physical mutagens are neutrons generated in nuclear reactors, which are producing large DNA deletions, but due to technical and safety problems, their application is limited. During the last two decades, ion beams generated by particle accelerators are more widely used due to their higher mutagenic potential (Watanabe 2001).

Double-stranded breaks of DNA induced by mutagenic agents are repaired by endogenous cell mechanisms, which usually results in heritable DNA recombination. Its extent depends on the type of tissue irradiated and the type and dose of radiation applied. Typically, the radiation doses causing 50% lethality (LD50) are applied, but it is arbitrary, and there are reports that milder treatments produce more desirable effects without deleterious mutations (Oldach 2011).

The ionizing radiation causes indiscriminate molecular lesions in plant DNA and its subsequent recombination by cellular DNA-repairing enzymes. In effect, the whole genome usually is affected, from localized deletions or insertions to gene replications, chromosome breaks, and their rearrangements, which may be accompanied by high rates of chromosome aberrations. A much milder mutagenic effect is seen in UV radiation, which has lower energy and usually does not break the DNA chain, but tends to produce purine or pyrimidine dimers, resulting in point mutations (Nakamura et al. 2021).

Chemical mutagens have gained popularity since they are easy to use, do not require any specialized equipment, and can provide a very high mutation frequency. However, they are carcinogenic, and strict safety standards must be applied when they are used. Chemical mutagens affect the genome by reacting with nucleobases in DNA molecules and may cause base deletions, insertions or substitutions. Compared to radiological methods, chemical mutagens tend to cause single base change (single-nucleotide polymorphisms) rather than whole genome recombination.

There are various mutagenic compounds differing in their mode of action. The alkylating agents covalently bind alkyl moieties to nitrogen, oxygen and phosphate groups in nucleobases, which in turn disrupts base pairing during DNA replication. For example, the most widely used ethyl methanesulfonate (EMS) selectively alkylates guanine, forming 6-O-ethylguanine. In effect, during DNA replication DNA-polymerase recognizes modified guanine as adenine and pairs it with thymine instead of cytosine in the complementary chain, which results in multiple base substitutions. Besides EMS, the other methylating agents used in mutational breeding include 1-methyl-1-nitrosourea, 1-ethyl-1-nitrosourea, methyl methanesulfonate, dimethyl sulphate, diethyl sulphate, 1-methyl-2-nitro-1-nitrosoguanidine, 1-ethyl-2-nitro-1-nitrosoguanidine, N,N-dimethylnitrous amide, and N,N-diethylnitrous amide (Leitao 2012).

Nucleobase analogs, like 5-bromouracil, maleic hydrazide, 5-bromodeoxyuridine, and 2-aminopurine can be incorporated into newly synthesized DNA molecule instead of the proper base. If occurs within the codon region, this results in multiple false triplet sequences and most often leads to gene knockout.

The other chemical mutagens are used less frequently in mutation breeding but are useful tools in genomic studies. Nitrous acid reacts with the amine group of adenine and cytosine by replacing them with hydroxyl groups by oxidative deamination. During DNA synthesis, DNA polymerase matches adenine to the deaminated cytosine and cytosine to the deaminated adenine, causing multiple base substitutions as in the case of alkylating agents (Zimmermann 1977).

The sodium azide mutagenic effect is not direct. It first converts O-acetylserine to β-azidoalanine in a reaction catalyzed by O-acetylserine(thiol)lyase in the cytosol. This metabolite is then transported to the nucleus where it reacts with DNA creating point mutations (Owais & Kleinhofs 1988).

The intercalating chemicals, like ethidium bromide, acridine orange, and actinomycin D intercalate between DNA bases in the native DNA helix. During DNA replication, DNA polymerase recognizes this distortion as an additional base and inserts an extra base opposite this stretched (intercalated) molecule (Leitao 2012).

The effect of base mutations depends on the place of the genome when they occur. Single base mutation (deletion, insertion, or substitution) in the introns or in regions of the genome with unknown function are usually silent. Mutations in the regulatory sequences of a gene, such as promoters, can alter levels of gene expression. Usually, such mutation has no effect on the amino acid sequence of the protein but may cause aberrant splicing of mRNA and affect its stability. Single or multiple base deletions or insertions in the coding region result in frameshift and translation of a protein different from the original one. A base substitution in the coding region can have no effect if the new codon encodes the same amino acid (synonymous substitution). The nonsynonymous substitution can lead to missense mutation or nonsense mutations. In the first case, the new codon encodes different amino acids, which are then built up into translated protein, frequently altering its function. The nonsense base substitution generates a codon, which does not encode any known amino acid. Such a codon can prematurely terminate the translation (premature stop codon) of a truncated protein. The single-nucleotide mutations induced by chemical mutagens can result in a loss-of-function by gene(s) but also in gain-of-function phenotypes if the mutation leads to a modified protein activity or affinity. For example, EMS-induced single nucleotide mutation in the wheat 5-enolpyruvylshikimate-3-phosphate synthase gene rendered it resistant to inhibition by the herbicide glyphosate, which resulted in a glyphosate-resistant phenotype (Aramrak et al. 2018).

A separate kind of mutational agents is mitotic inhibitors, like colchicine and oryzalin. They do not cause much recombination within chromosomes but change their copy number. However, duplicating whole sets of chromosomes may also generate epigenetic changes and modulate gene expression. As compared to diploids, polyploids may have higher yields and improved product quality, but also enhanced disease and stress resistance (Chen et al. 2020; Sattler et al. 2016).

The induced mutagenesis allowed to widen the genetic variability of cultivated plants, but its serious drawback is its randomness. The effect of mutational treatment in terms of desired changes in the plant genome and the phenotype cannot be predicted. Besides, in addition to desired traits, a high number of off-target mutations usually occur, which might have a negative impact on the phenotype. Thus, the key point in mutation breeding is the screening mutant population in order to select individuals with desired traits, e.g., disease resistance, crop quality, etc., followed by mutant confirmation, i.e., confirmation of the heredity of selected traits on selfed F_1 and F_2 populations and rejecting the false mutants (Oladosu et al. 2016). However, mutants' selection and confirmation are lengthy processes and in the case of woody plants having long juvenile periods may take up to several years. Thus, in terms of longevity, its advantage over traditional breeding is minimal. The more so that frequently beneficial traits are associated with deleterious mutations and their separation requires further cross-breeding and selections.

The progress in high-throughput sequencing allowed to shorten the mutant screening process using Targeting Induced Local Lesions in Genomes (TILLING) (Stemple 2004). In this method, after mutagenesis and mutant selection DNA is extracted from every single plant, and the key alleles are sequenced and analyzed for mutations. However, TILLING helps to speed up the breeding process, but does not eliminate the major drawback of random mutation, i.e., its unpredictability.

Despite its deficiencies, the random mutagenesis resulted in the breeding of many new, valuable cultivars of cultivated plants. During the past seventy years, many mutant varieties have been released worldwide, and it is foresighted that induced random mutations will continue to have applications in plant breeding. At present, the FAO/IAEA Mutant Varieties Database (https://www.iaea.org/resources/databases/mutant-varieties-database) contains more than 3,200 officially released mutant cultivars, and many more are being evaluated. In fruit crops, the prime strategy was to induce spur mutations of the well-established plant varieties in order to improve traits like fruit size and color, self-compatibility, growth vigor/dwarfism, seedlessness, and others (Sanada & Amano 1998; Sattar et al. 2021). However, only a very few fruit plant mutants expressed enhanced resistance to diseases (Yoshioka et al. 1999), which shows that random mutation breeding won't solve the problem of environmental pollution with plant protection chemicals.

The induced mutations cause extensive recombination of plant genomes, so it is par excellence genetic modification. However, plant cultivars obtained with this technique are explicitly excluded from the scope of Directive 2001/18/EC2 of the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms.

Site-directed mutagenesis

The attempts to eliminate major drawbacks of induced, random mutations and generate mutations at specific sites of the genome were done already in the late 1970s. The first method developed involved in-cell amplification of synthetic oligonucleotides with endogenous DNA polymerase and its ligation into the plant genome. The synthetic oligonucleotide, which is complementary to the selected genome site of the host cell but with a single nucleotide mismatched, deleted, or inserted, is transfected into the host cell, elongated by cell DNA polymerase, and ligated into the host genome by cell DNA repair mechanisms (Hutchison et al. 1978). However, the method worked well with bacteria and provided an excellent analytical tool for biochemistry and biology, but its application in eukaryotes, especially in plants, was not effective. The breakthrough comes when genetically-engineered, site-directed nucleases (SDNs) were developed: meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats/associated protein 9 (CRISPR/Cas9). Their application in genomic studies and breeding is covered by the broad term new genomic techniques (NGTs).

Meganucleases, also called homing nucleases, are endonucleases found in bacteria, phages, fungi, yeast, algae, and some plants. They have recognition sites specific to double-stranded DNA sequences of 12 to 40 base pairs long, and thus are highly specific for any given genome (Puchta et al. 1993). For applications in genome editing, their recognition site can be modified either by introducing a small number of variations to the amino acid sequence (Sussman et al. 2004) or by fusing protein domains from different enzymes (Smith et al. 2006). Such recombined meganucleases can modify chosen targeted sequence. However, the DNA-binding domain of meganucleases has no modular architecture; thus, it is difficult to engineer it. They are also prone to binding sequence degeneracy, which results in off-target DNA cleavage (Argast et al. 1998).

ZFNs are chimeric restriction enzymes composed of a sequence-specific DNA-binding domain linked to a DNA cleavage domain. The DNA binding domain usually contains Cys2His2-type zinc fingers, which are widespread in eukaryotic transcription factors. The individual finger binds a contiguous three-nucleotide subsequence; thus, the domain can be engineered to recognize a specific sequence of DNA triplets. The DNA nuclease domain usually consists of Fok1 endonuclease from *Flavobacterium okeanokoites*. ZFNs were first engineered in 1990s by Kim et al. (1996) and were applied for plant gene editing since 2005 (Zhang et al. 2010; Curtin et al. 2011). However, the individual zinc finger domains recognize DNA triplets, not single nucleotides, making the ZFN construct that recognizes a specific DNA somewhat limited. Due to that, off-target binding and DNA cleavage frequently occur. The method is also laborious and costly, so in the last years, more precise methods for gene editing are used, like TALENs and CRISPR/Cas (Modrzejewski et al. 2019).

TALENs are other types of chimeric enzymes consisting of a DNA-binding domain from a transcription activator-like (TAL) effector fused to a DNA cleavage domain. The TAL effectors are proteins secreted by pathogenic *Xanthomonas* bacteria during plant infection. In plant cells, TALs bind to promoter sequences in the host plant and activate the expression of plant genes that aid bacterial infection (Vivian & Arnold 2000). TALs have a domain of repeated highly-conserved amino acid sequences, which have a high affinity to specific nucleotides. This relationship between amino acid sequence and nucleotides allows for engineering specific DNA-binding domains (Boch 2011). As opposed to ZFNs, such an engineered binding site is specific not to a sequence of nucleotide triplets (codons) but to a sequence of individual nucleotides; thus, the occurrence of mismatched binding to DNA is very rare.

The CRISPR/Cas9 nuclease differs significantly from meganucleases, ZFNs, and TALENs as its DNA recognition domain is not a protein but RNA. CRISPR is a family of DNA sequences, found in the genomes of prokaryotic organisms, which are consisted of fragments of DNA (known as spacers) homologous to DNA sequences of some bacteriophages and plasmids. The CRISPR clusters are accompanied by a set of homologous Cas genes, which encode an enzyme that uses the CRISPR sequences as a guide to recognize and cleave specific strands of DNA that are complementary to the CRISPR sequence. The CRISPR/Cas plays a role in adaptive immunity against bacteriophages and plasmids in prokaryotes. During infection, CRISPR arrays are transcribed into small crRNAs (crisprRNAs) complementary to the target DNA, which then associate with Cas protein into crRNA-Cas complexes capable of recognizing and cleaving nucleic acids complementary to the crRNA (Barrangou 2015). The CRISPR sequence can be easily engineered to recognize any DNA sequence by introducing a short synthetic sequence of 20+ nucleotides. Of an array of Cas proteins, Cas9 is thought to be most suitable for crRNA-guided cleavage of foreign DNA. However, besides CRISPR guidance, Cas enzymes require also recognition of a protospacer adjacent motif (PAM) to cleave the nucleic acid at a predefined site. PAM is located downstream of the DNA sequence targeted by the guide RNA and consists of 2–7 nucleotides, depending on Cas origin. For example, for Cas9 from *Streptococcus pyogenes*, the PAM sequence is NGG (5' to 3'), but for Cas9 from *Streptococcus thermophilus*, it is NNAGAAW (Hanna & Doench 2020). Thus, for a given task a specific Cas enzyme must be selected. There are also attempts to engineer Cas9 for recognizing other PAM sequences (Kleinstiver et al. 2015). In the first experiments, the Cas9 derived from *Streptococcus pyogenes* has been used in constructing the CRISPR/Cas9 complex as a tool for gene editing (Jinek et al. 2012) and was later modified to increase target specificity and reduce off-target cleavage (Jaganathan et al. 2018). The most important advantage of CRISPR/Cas9 over other genome editing technologies is the ease of engineering its guiding sequences and its efficiency. At present, it is the most widely used technology used in plant breeding, but also in animal breeding and medicine, especially in gene therapy.

The mode of action of all sequence-specific nucleases is similar. They are transfected into the host cell as gene construct ligated into a plasmid or as mRNA. Upon protein expression by endogenous cell mechanisms, the nucleases bind to complementary DNA target and cleave it at the specified site creating a double-strand break. The cleavages are then repaired by the cellular DNA repair machinery either in a homologous or nonhomologous manner. In the nonhomologous repair (nonhomologous end joining), base insertions, deletions, and substitutions can occur, effectively leading to gene knockout. In the case of a homology-based process, a homologous donor DNA can be inserted at the site, leading to target gene modification or replacement (Osakabe & Osakabe 2015). The variant of CRISPR/Cas9 gene editing technology has also been developed, allowing single nucleotide editing without breaking double-stranded DNA. In this method, the catalytically-impaired CRISPR/Cas9 construct is fused with either cytidine or adenine deaminase. CRISPR-guided deamination of these nucleotides in the target DNA converts cytosine (C) to uracil (U) or adenine (A) to guanine (G), ultimately leading to a C-to-T or A-to-C substitution, respectively, without double-strand breaks (Komor et al. 2016; Gaudelli et al. 2017).

The development of genome editing technologies with the use of SDNs constituted a major breakthrough in biotechnology and molecular biology. It is confirmed by two Nobel Prizes bestowed for achievements in this area: in 1993 for fundamental contributions to the establishment of oligonucleotide-based, site-directed mutagenesis and its development for protein studies, and in 2020 for the development of the CRISPR/Cas9 genome editing technique. During the last two decades gene editing, especially CRISPR/Cas9-mediated, has been successfully applied in both animal and plant systems. High expectations in medicine are connected with CRISPR/Cas9-mediated gene therapy (Zhang 2021), cancer treatment (Zhao et al. 2021), xenotransplantology (Ryczek et al. 2021), and with development of new, more effective drugs and vaccines (Scott 2018). Gene-specific mutation technology is used to produce improved industrial enzymes for waste processing into fuels, thus contributing to the reduction of environmental pollution and alleviating the energy crisis (Zhu et al. 2022).

Gene editing of cultivated plants can speed up the breeding process to enhance agricultural productivity and ensure food security (Liu et al. 2021). It may also contribute to the adaptation of agriculture to climate changes and minimize its impact on the environment by breeding cultivars with increased resistance to pests and diseases and high tolerance to environmental stresses, especially drought and high temperatures (Sikora et al. 2011; Karavolias et al. 2021). The gene-editing technologies are especially important for woody fruit species in which classical breeding and random mutagenesis are very lengthy due to the long juvenile period. Due to progress in high throughput sequencing and functional genomics, the whole genomes of many fruit tree species have been completely sequenced and mapped and many more are being studied (Illa et al. 2011; Troggio et al. 2012; Chagné 2015; Zhang et al. 2021). Thus, the key genes conferring important phenotypes can be easily targeted and modified using site-directed mutagenesis techniques. Many promising clones with traits like increased resistance to diseases and drought and improved fruit quality have been obtained using this technology and are being tested in field trials (Ramirez-Torres et al. 2021).

The application of gene editing in medicine and industrial applications is widely accepted but arises public concern when it comes to edible crop species. It was very strongly expressed by the French agricultural organization Confédération paysanne, which brought the French Ministry for Agriculture, the Food Processing Industry and Forestry to the High Court (Conseil d'État) requesting revoking the French law that does not recognize organisms obtained by mutagenesis as genetically and specifically modified, to ban the cultivation and marketing of an herbicide-tolerant rape cultivar obtained by mutagenesis. Since the use of genetically-modified organisms is regulated by EU law, the French High Court has requested a preliminary ruling from the Court of Justice of the European Union, who on 25 July 2018 decreed that "organisms obtained by means of techniques/methods of mutagenesis constitute GMOs within the meaning of that provision of Directive 2001/18/EC2 of the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms". But at the same time, the Court also upheld the exemption of "conventional" mutation from the Directive, deciding that "genetically modified varieties obtained by means of techniques/methods of mutagenesis which have conventionally been used in a number of applications and have a long safety record are exempt from the obligations laid down in that provision" (CJEU 2018). This decision avoided havoc in administration and agriculture, since covering all the plants obtained by mutagenesis with the obligations of the directive would require re-registering a several thousand plant cultivars already in cultivation throughout the EU. But at the same time this decision by the Court is controversial because it makes a distinction between similar mutants based only on the method by which they were obtained, but not on the biology. Gene edition both by chemical mutagens and SDNs can result in single nucleotide mutation, usually leading to gene knockout. The impact of both types of mutation on plant phenotype is identical, and there is no transgenesis or cisgenesis involved. But site-directed mutagenesis has an advantage because it causes mutation at a precisely defined site within the gene, with very few, if any, off-target events

whereas chemical mutagens cause random, multiple single-point mutations both within the target gene and within other parts of the genome. Even more recombinations of plant genomes are caused by physical mutagens. Nevertheless, mutants obtained by site-directed mutagenesis are considered inferior and "not safe", thus requiring special regulation for their release, whereas such regulations are not applied to "classical", randomly-induced mutants.

The decision of the Court raised controversies and initiated fierce discussion among researchers, breeders, farmers, laymen, and other stakeholders. The search of Google for "C-528/16" done on May 10, 2022, yielded approximately 82,000 results, and a search of Google Scholar at the same time yielded 520 results, mainly research papers. The comments on the Internet and the research papers were not only in English or other languages of EU Member States but also in other languages, including Chinese, Japanese, Korean and Russian. This shows that the decision of the Court of Justice of the EU is of worldwide interest.

The decision of the Court and the following discussion prompted the Council of the EU to request from the European Commission a throughout analysis of the status of NGTs under the Union's law in light of the Court of Justice ruling in Case C-528/16. It also asked the Commission to submit a proposal accompanied by an impact assessment, if appropriate in view of the outcomes of the study.

The report from the study was published on 29 April 2021 (EC 2021). Its main findings are below.

- There was a rapid development of NGTs during the last 20 years, but the main progress was done outside the EU.
- In many EU Member States there is considerable interest in NGT-related research, but the current regulatory framework has a negative impact both on public and private research and innovation in that area.
- NGT products may likely contribute to sustainable agri-food systems in line with the objectives of the European Green Deal and Farm to Fork Strategy. But potential NGT applications in the agricultural sector should not undermine other aspects of sustainable food production, e.g., organic agriculture and biodiversity.
- The implementation and enforcement of the current EU regulatory system are problematic because the available methods are not fit to detect genome modification in NGT-derived products that do not contain any foreign genetic material.
- NGTs would allow the development of new medicinal products and therapies.
- As concluded by the EFSA Panel on Genetically Modified Organisms (EFSA 2020), no new hazards in plant breeding are connected with established genomic techniques as compared with conventional breeding. Plant products with similar risk profiles can be obtained both with conventional breeding techniques, targeted mutagenesis, and cisgenesis.
- For other NGTs or for applications in animals and microorganisms, the scientific knowledge is still limited or lacking, especially on safety aspects.

Below are the main conclusions of the study.

- There are strong indications that the applicable legislation is not fit for the purpose of some NGTs and their products, and that it needs to be adapted to scientific and technological progress. It may not be justified to apply different levels of regulatory oversight to similar products with similar levels of risk, as is the case for plants conventionally bred and obtained from certain NGTs.
- The follow-up to the study should confirm whether adaptation is needed and, if so, what form it should take and which policy instruments should be used in order for the legislation to be resilient, future-proof, and uniformly applied.

As a follow-up, the European Commission plans to initiate a policy action on plants produced by targeted mutagenesis and cisgenesis, which will involve an impact assessment including a public consultation (https://food.ec.europa.eu/plants/genetically-modified-organisms/new-techniques-biotechnology/ec-study-new-genomic-techniques_en).

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