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REVIEW

Fruit crops in the era of genome editing: closing the regulatory gap

Derry Alvarez[1] · Pedro Cerda-Bennasser[1] · Evan Stowe[2,3] · Fabiola Ramirez-Torres[2,3] · Teresa Capell[1] · Amit Dhingra[2,3] · Paul Christou[1,4]

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Abstract

The conventional breeding of fruits and fruit trees has led to the improvement of consumer-driven traits such as fruit size, yield, nutritional properties, aroma and taste, as well as the introduction of agronomic properties such as disease resistance. However, even with the assistance of modern molecular approaches such as marker-assisted selection, the improvement of fruit varieties by conventional breeding takes considerable time and effort. The advent of genetic engineering led to the rapid development of new varieties by allowing the direct introduction of genes into elite lines. In this review article, we discuss three such case studies: the Arctic® apple, the Pinkglow pineapple and the SunUp/Rainbow papaya. We consider these events in the light of global regulations for the commercialization of genetically modified organisms (GMOs), focusing on the differences between product-related systems (the USA/Canada comparative safety assessment) and process-related systems (the EU "precautionary principle" model). More recently, genome editing has provided an efficient way to introduce precise mutations in plants, including fruits and fruit trees, replicating conventional breeding outcomes without the extensive backcrossing and selection typically necessary to introgress new traits. Some jurisdictions have reacted by amending the regulations governing GMOs to provide exemptions for crops that would be indistinguishable from conventional varieties based on product comparison. This has revealed the deficiencies of current process-related regulatory frameworks, particularly in the EU, which now stands against the rest of the world as a unique example of inflexible and dogmatic governance based on political expediency and activism rather than rigorous scientific evidence.

Keywords Apple · Papaya · Pineapple · Genome editing · Commercialization

Introduction

Fruit crops have traditionally been improved by conventional breeding, which involves selecting plants with desirable traits over many successive generations to achieve incremental improvements in phenotypes such as fruit size, yield, nutritional properties, and aroma/taste (Moreno-González and Cubero 1993). The introduction of new traits, such as disease resistance, requires the introgression of the corresponding alleles into elite lines that have already been honed by many generations of selection. For example, a high-yielding elite line is crossed with a disease-resistant variety followed by repeated backcrossing with the elite parent, aiming to preserve as much of the genetic material from the elite variety as possible without losing the new resistance allele. It takes many generations to somewhat restore the elite background, and in species with a long juvenile phase it may take several decades for the selectable phenotype to emerge in each generation. Compounding breeding difficulties is

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Derry Alvarez and Pedro Cerda-Bennasser contributed equally to the article.

✉ Amit Dhingra
adhingra@wsu.edu

✉ Paul Christou
paul.christou@udl.cat

[1] Department of Plant Production and Forestry Science, University of Lleida-Agrotecnio Center, Lleida, Spain

[2] Department of Horticulture, Washington State University, Pullman, WA 99164, USA

[3] Molecular Plant Sciences Program, Washington State University, Pullman, WA 99164, USA

[4] ICREA, Catalan Institute for Research and Advanced Studies, Barcelona, Spain

the obligate outcrossing nature of many fruit-bearing crops, which makes it impossible to recover the original genotype and phenotype. Therefore, the chance selection of a desirable phenotype in an elite cultivar is highly valuable. Development of resistance to apple scab illustrates this point very well where the crosses between a wild type and an elite line of apple were initiated in the 1950s and the derivative cultivars are still lacking in desired fruit quality traits (Hough et al. 1953; Schouten et al. 2006). The latter has been addressed to a degree by the development of marker-assisted selection (Lande and Thompson 1990), in which molecular markers linked to the desired trait can be selected at an earlier developmental stage, but this does not provide a shortcut to the generations of backcrossing required to achieve successful introgression (Semagn et al. 2006). However, it is possible to overcome extensive juvenility in woody species via transgenic expression of the FLOWERING LOCUS T (FT) and other MADS-box genes, in an approach called Fastrack breeding. An example of this is the 'Pinova' apple, which has been transformed to express a MADS-box gene from silver birch (*Betula pendula*), *BpMADS4* imparting an early flowering phenotype (Elo et al. 2007; Flachowsky et al. 2007). While this presents a practical approach, to obtain a non-GMO product through Fastrack breeding, the gene responsible for early flowering will need to be segregated out.

From the 1950s onward, several new strategies were developed to provide faster access to genetic diversity. Rather than waiting for the serendipitous emergence of disease-resistant varieties of a fruit species, researchers and agronomists developed various wide crossing methods in which sexually incompatible species formed hybrids that were rendered fertile by blocking cell division in culture, resulting in polyploidy and the restoration of productive meiosis. In some cases, these new fertile interspecific and intergeneric hybrids were derived from sterile hybrids that formed in nature (Stalker 1980), but more aggressive forced hybridization approaches were also developed including chromosome addition/removal (Thomas 1993) and somatic hybridization by protoplast fusion to bring together species that would otherwise be unable to form offspring (Sink et al. 1992). Further genetic diversity was introduced by using chemical mutagens, radiation or transposons, expanding the number of potentially valuable alleles directly in the elite lines (Lapins 1983). These methods were limited by the pool of genetic information available in the fruit crop or species close enough to form hybrids.

The next major step forward was the advent of transgenic plants, allowing the direct introduction of specific genetic constructs from any source (Gasser and Fraley 1989). Several methods were developed concurrently, including transformation by the soil bacterium *Agrobacterium tumefaciens*, physical gene transfer methods such as particle bombardment and electroporation, and chemical transfection methods for protoplasts. For the first time, this extended the available gene pool beyond plants, but all methods involved some form of in vitro culture step, with many fruit species exhibiting recalcitrance to transformation and/or regeneration. In species and cultivars that were amenable to both, it became possible to introgress new genes directly into elite varieties in a single generation, eliminating the need for repetitive backcrossing (Kohli and Christou 2008). However, the advent of genetic engineering also brought with it a greater regulatory burden, because for the first time these new plant varieties carrying genes from other species were regarded as different, in a specific legal context, from other crop varieties produced by so-called natural methods (Masip et al. 2013; Ammann 2014). This provided the first inkling of the dichotomy between regulating the product and regulating the process because it raised the prospect of a genetically modified plant being treated as distinct from a natural variety even if both were genetically identical. Initially, this theoretical consideration was not thought to have any realistic practical implications because all genetic engineering strategies, even if they introduced genes from the same species, left some footprints of foreign DNA that advertised their status as a genetically modified organism (GMO), such as selectable marker genes, parts of the vector backbone, or parts of the *A. tumefaciens* T-DNA (Twyman et al. 2002).

In the last decade, the arrival of genome editing has resulted in another leap forward in breeding technology (Jansing et al. 2019; Ghogare et al. 2020). Genome editing involves the expression of specialized nucleases that introduce double-strand breaks (DSBs) at precise and pre-selected targets in the plant genome. The inaccurate repair of these DSBs results in the formation of indels that inactivate the targeted gene, although the provision of donor DNA matching the flanks of the target site can achieve the integration of new sequences (analogous to transgene insertion, but more controlled) or the replacement of one sequence with another. Importantly, these processes leave no other footprints behind and, at the sequence level, the indel mutants are indistinguishable from natural mutations or those induced by chemicals or radiation (Pérez-Massot et al. 2013; Zhu et al. 2017). Mutations induced by chemicals or radiation are usually hemizygous. In such lines, homozygosity may be obtained by closely observing filial segregates and performing the necessary back crosses to fix the mutated gene of interest. The true utility of site-directed mutagenesis via gene editing, however, lies within its ability to target multiple copies of a gene, including homoeologous ones in polyploids. More recently, variants of the genome-editing method have been developed for single-nucleotide replacement, allowing the precise swapping of one base for another in a DNA strand (Monsur et al.

2020). These technologies expose the illogical nature of current regulatory frameworks based on the process rather than the product because it is impossible to distinguish a natural plant variety from one generated in the laboratory (Hartung and Schiemann 2014). Furthermore, there is evidence from a number of natural plants (including at least two major crops) that the evolution of some plant families has involved the integration of *Agrobacterium* genes, leaving ancient T-DNA footprints that again blur the boundary between 'natural' and 'engineered' species (Quispe-Huamanquispe et al. 2017). In this article, we look at three case studies of fruit crops developed using modern breeding methods, consider whether the same goals could have been achieved using alternative technologies, and discuss their regulation in different jurisdictions as a means to highlight the unworkable current legal framework for the definition and control of new fruit crop varieties.

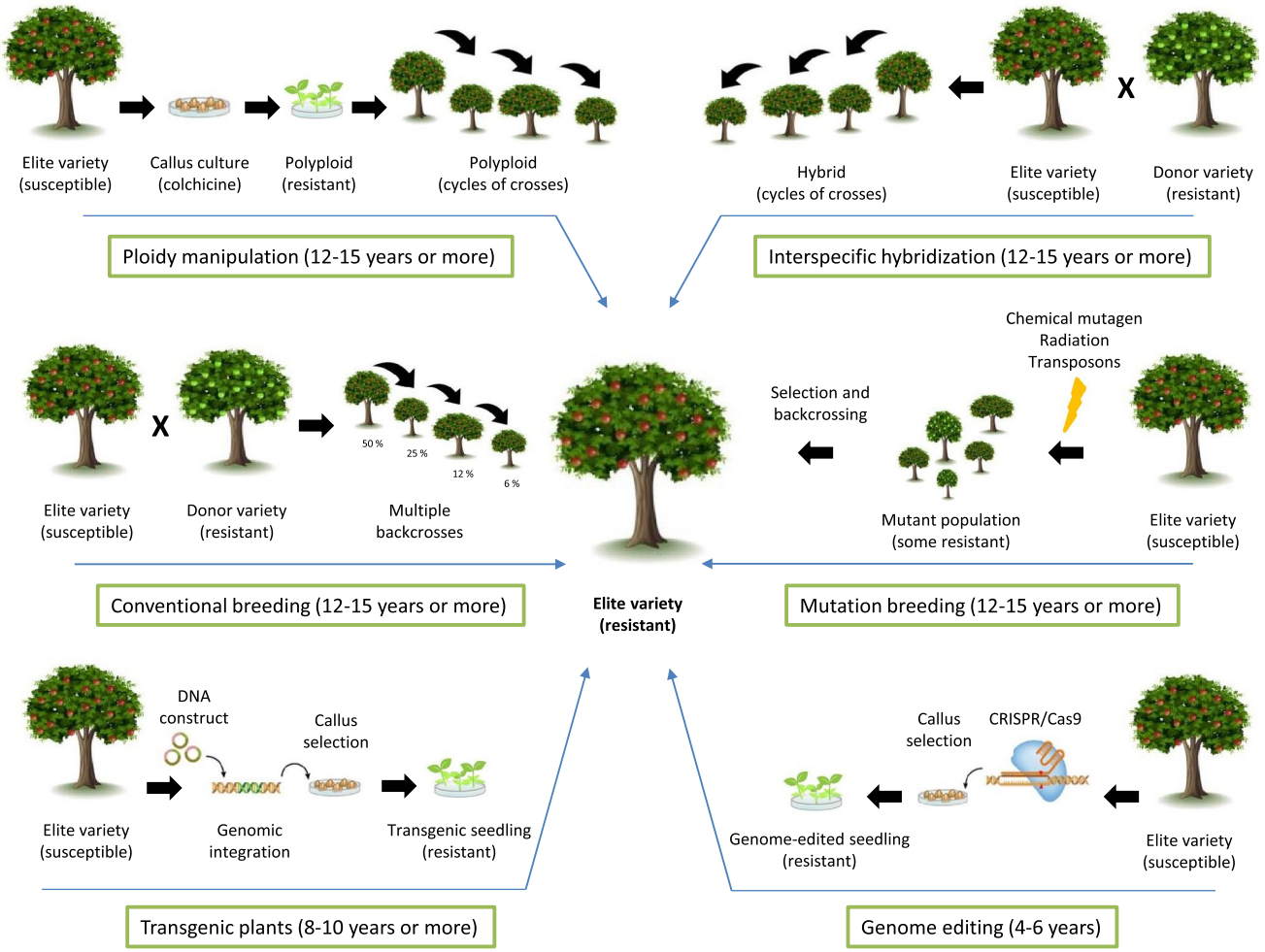

Fig. 1 Comparison of different methods and timelines to develop fruit crops with a desirable new trait, such as disease resistance. Top left: ploidy manipulation involves the use of chemicals such as colchicine to induce polyploidy in culture, followed by regeneration and crossing. Top right: interspecific hybridization involves the natural or forced hybridization of different species, followed by crosses to one or both parents. Forced hybridization by protoplast fusion would also involve a callus culture and regeneration step. Middle left: convention breeding involves the crossing of different varieties followed by multiple generations of backcrossing to reinstate the elite background while retaining the new trait. Middle right: mutation breeding involves the mutagenization of a population, the selection of desired mutants for crossing. Bottom left: transgenic plants often allow the direct introduction of desirable traits into an elite background, resulting in a short biological process (green arrow). However, the regulatory approval of such varieties takes much longer (red arrow). Bottom right: in the case of genome editing, the short biological process (green arrow) is sufficient in regulatory jurisdictions that follow a US-type system but a length regulatory approval process (red arrow) is required in the EU, which currently follows a strict process-based regulatory system

Different breeding technologies applied to fruit crops

Conventional breeding

The genetic improvement of fruit crops requires several cycles of selection for better-performing genotypes derived through the process of chromosomal recombination (Janick 1998; Moose and Mumm 2008). The major bottleneck in conventional breeding is the large number of crosses required to represent as many genetic recombination events as possible (Fig. 1). In perennial fruit crops, seedlings enter a prolonged juvenile phase before the progeny can be phenotyped for desired traits, which means the process of recurrent selection can take several decades (Guzman and Dhingra 2019; Kaiser et al. 2020). For example, a series of natural hybridization and selection events in the evolution of apple (*Malus* spp.) has produced cultivars with diverse quality traits such as sweetness, color, firmness, and size (Cornille et al. 2014; Kaiser et al. 2020). Cultivar development has been accelerated by the incorporation of molecular markers for early selection, and genomic technologies such as genome-wide association studies (GWAS) to identify quantitative trait loci (QTLs) more efficiently (Brachi et al. 2011; Iwata et al. 2016; Moose and Mumm 2008). Examples of traits that have been introduced using these modern conventional breeding approaches include higher nutritional value, early maturity, increased yield, cold resistance, superior taste, seedlessness, and disease resistance in mandarin (Omura and Shimada 2016), cucumber (Feng et al. 2020), peach, strawberry, apple, and banana (Bouis 2002; Moose and Mumm 2008). With the availability of pangenomes and an increasing understanding of genotype–phenotype relationships combined with Fastrack breeding, it may be feasible to develop desirable cultivars at a faster rate.

Chemical, radiation and transposon mutagenesis

Conventional breeding is limited to the genetic diversity available in sexually compatible plants (International Atomic Energy Agency 2001; Te Beest et al. 2012). The genetic diversity of otherwise 'closed' populations can be increased by treating seeds, seedlings, cultured cells, or even whole plants with agents that induce new mutations. Seeds are preferred targets because the embryo has a small number of cells, so mutagenesis is easier to control and results in fewer chimeric plants (Lamo et al. 2017). The frequency and structure of new mutations depend on the mutagen type and the exposure (Mba et al. 2010). However, the mutations generated are random and large populations (mutant libraries) must be screened to identify desirable events (Kumawat et al. 2019). The most significant advantage of mutagenesis is that it obviates the need for genetic segregation while incrementally improving an elite selection.

The earliest deliberate mutagenesis method applied directly to plants was high-energy radiation in the form of X-rays (Lamo et al. 2017). This approach is still used today, along with other sources such as fast neutrons, ionizing radiation and gamma rays, triggering direct DNA damage as well as indirect effects caused by oxygen radicals, and accordingly generating diverse mutations ranging from single-nucleotide replacements to large deletions and chromosomal aberrations (Predieri 2001). For example, gamma rays have been used to improve heat tolerance in pineapple (Lokko and Amoatey 2001), self-fertility in sweet cherry, fruit color in apple, bunch size and early growth in banana, dwarf stature in papaya, disease-resistance in pear and strawberry, and early growth in grapevine (International Atomic Energy Agency 2001).

Chemical mutagens generate DNA adducts that lead to inaccurate repair, predominantly resulting in point mutations, and by adjusting the dose it is possible to generate simultaneous mutations in different genes (International Atomic Energy Agency 2001). Alkylating agents such as ethylmethanesulfonate (EMS) are widely used in fruit crops (Kodym and Afza 2003; Mba et al. 2010). For example, EMS has been used to induce mutations in banana, cucumber and tomato (Novak 1990; Wang et al. 2014; Binti et al. 2015). Chemical mutagenesis is efficient in whole plants and seeds but less efficient in tissue culture due to toxicity (Lamo et al. 2017).

Transposons are naturally occurring segments of DNA that can 'jump' along the genome and insert themselves in intergenic or genic regions. Transposon-mediated mutagenesis is a process in which transposable elements are induced to mobilize during early development. The objective is often to generate a saturation library where every gene is interrupted in at least one individual of the resulting population (Barquist et al. 2016). This common functional genomics approach has also been used to improve crop traits, although few applications have been reported in fruit crops. In tomato, heterologous transposable elements have been introduced from maize to induce the formation of insertional mutants (Cooley et al. 1996; Chaudary et al. 2019), which represents a hybrid approach between transposon mutagenesis and transgene insertion. However, endogenous *Rider* transposons have also been used for the same purpose (Roldan et al. 2017).

Polyploidy and interspecific hybridization

Natural polyploidy has been critical for the evolution, adaptation and speciation of plants (Ramírez-Madera et al. 2017; Ramsey and Schemske 1998). Polyploidy can also be induced in fruit crops by treatment with chemicals such as

colchicine that block mitosis without inhibiting DNA replication, leading to a doubling of the chromosome number. This phenomenon is known as autopolyploidy because the doubled set of chromosomes arises from one source. Unlike animals, where chromosome aberrations are usually lethal, plants show a much greater tolerance of gene dosage effects and polyploidy is often beneficial, for example conferring stress resistance (Zhu Hongju 2018). In grapevine, triploid and tetraploid lines generated by exposure to 0.05% colchicine produced larger, higher-quality berries, without changes in color, acidity, or soluble carbohydrates. However, only the tetraploids remained vigorous in growth (Notsuka et al. 2000). In dessert apples, triploids are often sought in breeding programs for their propensity to have larger fruiting bodies (Spengler 2019).

Interspecific hybridization or wide crosses have also been used to improve fruit crops. The methods to achieve this include the natural formation of hybrids by sexual reproduction, as seen with many citrus varieties (Wu et al. 2014), as well as more aggressive methods, such as somatic hybridization (Ohgawara et al. 1991). In perennial crops, if the hybrids are fertile, interspecific hybridization requires several rounds of backcrossing with one or both parents to eliminate undesirable background effects, and the screening of desirable progeny is laborious (Abbott 1992). This approach has been used to introgress scab resistance in apples but took more than four decades (Crosby et al. 1992). Similarly, the development of elderberries with higher sugar content and antioxidants by interspecific hybridization and backcrossing took a decade (Mikulic-Petkovsek et al. 2016). Where the hybrids are sterile, the induction of polyploidy can restore the ability of germ cells to undergo productive meiosis and therefore render the hybrid fertile, a phenomenon known as allopolyploidy because the doubled chromosomes arise from more than one source. Similarly, polyploidy achieved by protoplast fusion is a form of allopolyploidy because two complete sets of chromosomes, one from each donor species, are introduced into one cell (Zhu and Liu 2018).

Genetic engineering

Genetically engineered plants are modified by the insertion of one or more genes not limited by species or kingdom, allowing the introduction of desirable traits directly in an elite background in a single generation. The aim of genetic engineering is to improve important traits such as fruit yield or quality, or resistance to biotic or abiotic stress (Jhansi Rani and Usha 2013; Ricroch and Hénard-Damave 2016; Yabor et al. 2020; Yau and Stewart 2013). The first approved transgenic fruit (GMO) was the Flavr Savr tomato developed by Calgene, a biotechnology company later acquired by Monsanto (Redenbaugh et al. 1992). The Flavr Savr variety expressed an antisense RNA to suppress the expression of β-polygalacturonase, the enzyme responsible for pectin degradation and therefore fruit softening, giving the fruits a longer shelf life. Flavr Savr was approved by the US Food and Drug Administration (FDA) in 1994 but was withdrawn in 1997 when the company succumbed to financial difficulties, in part precipitated by the poor choice of genotype selected to create the Flavr Savr tomato (McHughen 2001). After tomato, the next transgenic fruit developed was papaya varieties (Sunset and Kapoho) in which the gene coding for capsid protein from Papaya Ringspot Virus (PRSV) was inserted into the papaya genome. In 1998 the first virus-resistant transgenic papaya was released (Gonsalves 2006). More recently, a transgenic non-browning apple has been approved for release in the USA based on the same principle (Carter 2012; Xu 2013; Igarashi et al. 2016; Stowe and Dhingra 2021). The transgenic approach has been used to improve several other traits in apple, pineapple, papaya and banana, such as fruit quality and firmness, growth habit, and tolerance to abiotic stress (Gonsalves 2006; Igarashi et al. 2016; Sreedharan et al. 2013; Yabor et al. 2020). Currently, the only transgenic fruit crops approved in the US are a papaya ringspot virus-resistant papaya (Gonsalves 2006), a plumpox virus-resistant plum (Scorza et al. 2012), the non-browning apple described above (Xu 2013) and a Pinkglow pineapple variety (FDA 2018). Three of these events are described in the case studies that follow.

Genome editing

Genome editing allows the introduction of mutations at predefined sites by targeting a particular unique sequence with a guided nuclease. Various genome editing platforms have been described, but the three that have been used in fruit crops are zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas9 system (Ghogare et al. 2020; Shukla et al. 2009; Zhang et al. 2013). The principle of ZFNs and TALENs is similar in that both are based on the type IIS restriction endonuclease FokI, in which the sequence-dependent DNA-binding domain and endonuclease domain are physically and functionally separate. Accordingly, the endonuclease domain cuts at a non-specific sequence a few nucleotides downstream of the specific target site. By replacing the FokI DNA-binding domain with a series of DNA-binding zinc finger modules (ZFNs) or TAL effector domains (TALENs), the nuclease can be designed to target any sequence of choice in the plant genome (Zhang et al. 2013). Unlike the protein-guided ZFNs and TALENs, the CRISPR/Cas9 system is based on a nuclease (Cas9) that recognizes a very short and therefore abundant sequence (3–8 nt long) known as the protospacer adjacent motif (PAM). However, the nuclease is guided to a more specific target by a guide RNA (gRNA), which is a complementary sequence next to the PAM. The gRNA

is ~ 20 nucleotides in length. The CRISPR/Cas9 system is more versatile than the others because gRNAs are much easier to design and produce than ZFN and TALEN modules, and multiple gRNAs can be used to target different genes simultaneously (Bortesi et al. 2016; Armario Najera et al. 2019).

Zinc finger nucleases (ZFNs) have been used to edit selectable markers in apple and fig (Peer et al. 2015), whereas TALENs have been used to enhance traits in several fruit and vegetable crops (Khan et al. 2017). However, these systems have only been used to a limited extent in fruit trees due to the complex principles of construct design (Carroll 2011). CRISPR/Cas9 has been widely used to edit multiple fruit crops (Zhou et al. 2020). For example, resistance to abiotic stress has been improved by using CRISPR/Cas9 in tomato, banana, grapevine, papaya, watermelon and cacao (De Toledo et al. 2016; Tashkandi et al. 2018; Tian et al. 2018; Wang et al. 2017; Yin et al. 2018). CRISPR/Cas9 in has also been used for the domestication of tomato, cucumber, groundcherry and kiwifruit varieties (Hu et al. 2017; Lemmon et al. 2018; Li et al. 2018a, b; Varkonyi-Gasic et al. 2019; Zsögön et al. 2018).

A comparative assessment of the approaches of crop improvement indicates that genome editing of elite cultivars requires the shortest duration to reach the retail market without the regulatory processes (Fig. 1). While conventional, polyploid and mutagenesis breeding take 12–15 years to select a desirable genotype, it should be noted that the multi-generation (vegetative), multi-location and multi-year agronomic and post-harvest evaluation require another 7–10 years before a variety can be released. A newly released variety will then need to scale up before reaching the market extending the time from the first cross to the market shelf to about 40 years.

Regulation of crops produced using new technology

The laws and regulations governing food are intended to ensure safety and quality throughout the food production and distribution chain (FAO 2020). International legislation (Table 1) has evolved in response to new technologies, but in some jurisdictions this has occurred in a politically expedient rather than science-based manner, resulting in unnecessary complexity and a lack of international harmonization (Farre et al. 2011). The lack of a standard approach spanning international boundaries causes difficulties in the enforcement of international agreements and disrupts international trade (Masip et al. 2013; Pérez-Massot et al. 2013; NASEM 2016).

The two main approaches for the safety assessment of novel foods (including the products of genetic engineering and genome editing) focus on the product or the process, respectively (Mayer 2009). Jurisdictions have tended to follow either the EU model, which is based on the production process or the US model, which is based on the assessment of features in the final product (Fig. 2). However, some hybrid models are now beginning to emerge. The regulation of crops produced by genetic engineering g in the USA and Canada has built on pre-existing regulations covering conventional crops, and is based on the principle of "substantial equivalence" in which the new crop is compared to its nearest natural equivalent (Ahmad 2014; Acosta 2014). In the US crops generated by genome editing are classed along with conventional varieties if the genome editing approach is used to introduce simple mutations, but are classed as GMOs if the genome editing approach was used to introduce foreign genetic material. Canada has yet to establish a formal policy on gene editing. In the US, the FDA has not yet announced how it will regulate editing. The USDA allows one edit (be it a double-strand break or a nucleotide) to be made. Thus, edited gene families and homoeologs are regulated articles (US does not use the term GM), not conventional. On the other hand, the US is unique in that it now allows cisgenes to be considered conventional, even if transferred by rDNA technology. However, if the edit results in resistance to a disease or pest, the EPA (which has presented a draft regulation) will be involved. An indel will be allowed as conventional as long as an allele with the exact nucleotide sequence exists in a sexually compatible relative. Alternatively, entire removal of a gene such that no mRNA is produced will be allowable. Cis genes will be allowable as long as the level of expression does not exceed what is in the sexually compatible relative, or the timing and tissue of expression are not altered over what happens in the sexually compatible relative (Drs A. McHughen and H. Quemanda—personal communication; anonymous reviewer).

Other countries in the Americas, such as Brazil and Argentina, have introduced specific new federal statues for the regulation of crops designated as GMOs and also classify genome-edited crops using the same dual approach although there are questions as to how much DNA can be added before it becomes a GMO. Of note is the fact that Brazil and Argentina put in place regulations for genome-edited crops before the USDA did, while the FDA and EPA still do not have theirs in place. The CTNBio's Normative Resolution nº16 (RN16) was published on 15 January 2018 (RESOLUÇÃO NORMATIVA Nº 16, 2018) and it laws covers products generated through new Breeding Technologies, including Genome Editing. Brazilian regulations for genome-edited crops follow a case by case approach. Thus a product generated by genome editing may be considered conventional if recombinant DNA/RNA is absent in the progeny, or if the genetic elements could be obtained by conventional breeding; such products may be considered as

Table 1 Regulatory agencies and basis of regulation in different jurisdictions

	European Union	United States	Canada	Argentina	Brazil	China	India	Australia	Japan
Regulatory agencies	EFSA (European Food Safety Authority)	USDA-APHIS, FDA, EPA	CFIA, Health Canada	SAGPA, BD, SENASA, AMD	CNBS, CTNBio, CIBio	MARA (Chinese Ministry of Agriculture and Rural Affairs)	GEAC (Genetic Engineering Appraisal Committee)	OGTR (Office of the Gene Technology Regulator)	MOE (Ministry of Environment
Basis of regulation	Regulation No. 1829/2003, or by national competent authorities under Directive 2001/18/EC, which regulates the intentional release of GMOs into the environment	Plant Protection Act (PPA) of 2000, The Federal Food and Drug Cosmetic Act (FFDCA) of 1938, Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) of 1996, State Law	Canadian Food and Drugs Act of 1985, Federal Regulatory Framework for Biotechnology in 1993	Secretaria de Agricultura, Ganaderia y Pesca Resolucion 173/2015	Biosafety Law Law No. 11,105 of 2005	"Administrative Rules for Safety of Agriculture GMOs" (issued in 2001 and revised in 2017)	The regulatory framework for GE crops, animals, and products in India is governed by the EPA of 1986 and the 'Rules for the Manufacture, Use/Import/Export and Storage of Hazardous Microorganisms/Genetically Engineered Organisms or Cells, 1989´	Gene Technology Act (2000) and Food Standards Australia New Zealand Act 1991	Cartagena Law 2003, Food Safety Basic Act 2003, Food sanitation Law 1947 and Act on Safety Assurance and Quality Improvement of Feeds 1953

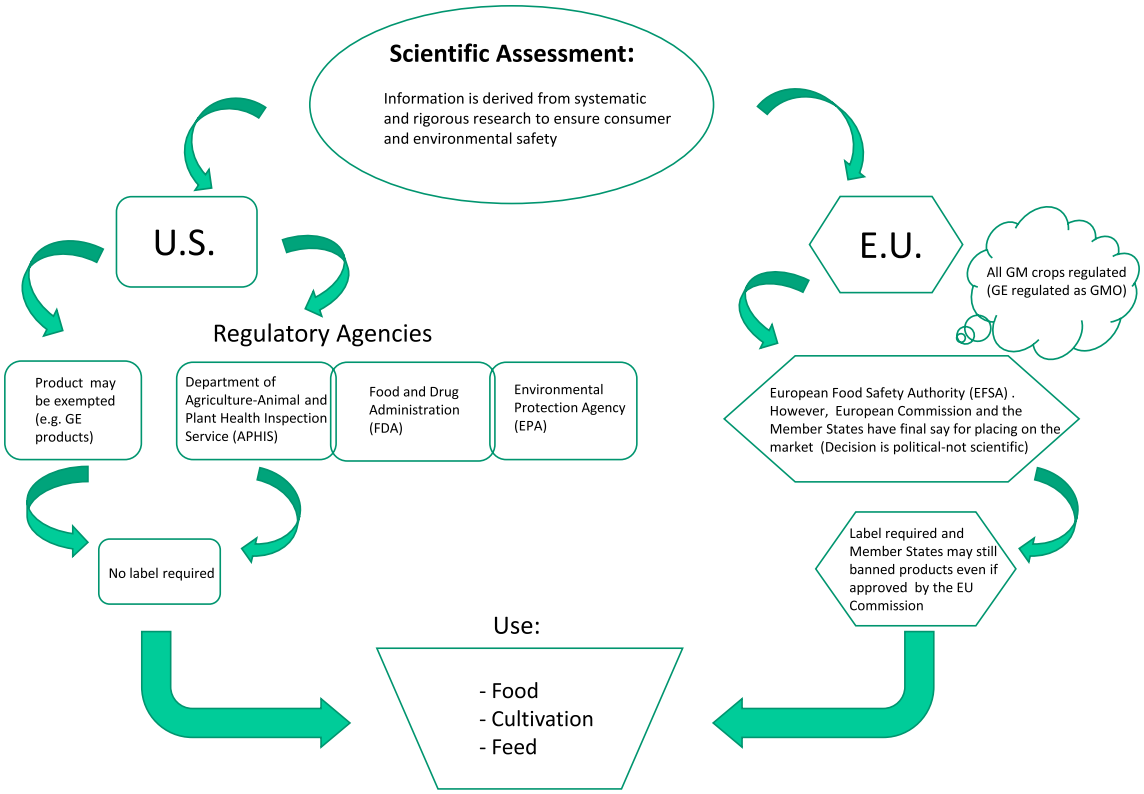

Fig. 2 Differences in the basis of GMO regulation between the US and the EU

conventional if the induced mutations could also be obtained by older techniques, such as radiation or chemical mutagenesis, or even the presence of induced mutations that could occur naturally (Entine et al. (in press); Whelan and Lema 2015).

In Europe, crops designated as GMOs are much more strictly regulated than in the Americas, and although such crops are not banned de jure, the complexity and hostility of the legislation ensures that a de facto moratorium is in place which limits production to a very small number of sites. GMOs are covered by Regulation (EC) no. 1829/2003 on genetically modified food and feed and Directive 2001/18/EC on the release of GMOs into the environment. Following the evaluation of food-related and environmental safety risks by the European Food Safety Authority (EFSA), the final decision on whether to allow cultivation for market production is determined by the European Commission with individual Member States allowed a veto for any reason, not necessarily based on scientific principles (NASEM 2016). Failing that, petitions for approval go to the Regulatory Committee, which usually gives an unfavorable opinion for the same reason. Then it goes to the ministers, and 2/3 must vote for approval, something that has only happened twice. If 2/3 do not vote positively, or if 3 months transpire without a decision, the petition returns to the EC and the cycle repeats itself (Sabalza et al. 2011).

This means that decisions are often made for political reasons and can override a positive evaluation from EFSA (Sabalza et al. 2011; Masip et al. 2013). The regulatory system is based on the process, so any GMO is classed separately from the products of conventional breeding no matter how similar it is to a conventional variety. For example, a hybrid generated by protoplast fusion involving two distantly related species, followed by chromosome manipulation in vitro and extensive chemical mutagenesis would still be classified as a natural variety, whereas a transgenic plant with a single base change would be classified as a GMO and would attract much greater regulatory scrutiny. Accordingly, crops generated by genome editing are regulated as GMOs regardless of the nature of the genetic change, and under current regulations this would even apply to new crop varieties generated using CRISPR/Cas9 ribonucleoprotein particles, which are DNA-free mutagenesis methods conceptually no different to chemical mutagenesis but a whole lot more accurate.

Asia, China and India grow GMO crops on a large scale and follow a broadly process-based classification system based on the EU model, but in both cases there is legislation in development that would assert a hybrid model especially

regarding the classification of genome-edited crops on the basis of product characteristics rather than process, closer to the dual system that applies in the Americas. The Chinese Ministry of Agriculture and Rural Affairs implements policies related to agriculture, rural areas, and rural residents, and regulates crops, animal husbandry, fisheries, agricultural mechanization, and the quality of agricultural products, whereas the Department of Science, Technology and Education (Agricultural GMO Biosafety Management Office) controls agricultural science and technology, system reform, research and development, technology imports, and commercialization (Xinrong et al. 2018). In India, the regulation of genetically modified and edited crops is covered by the 1986 Environment Protection Act under the oversight of the Genetic Engineering Appraisal Committee (GEAC) due to the absence of specific regulations and operational infrastructure.

In Japan, GMO crops are regulated using a product-based system like the USA and Canada, and genome editing is assessed using the same dual-classification approach (USDA 2013). In Australia, GMO crops are regulated similarly to Argentina and Brazil through the Gene Technology Act 2000 and are overseen by Food Standards Australia New Zealand (FSANZ) which applies an EU-like process-based system, but genome-edited crops that carry simple mutations are exempt (Office of the Gene Technology Regulator 2020). The EU is therefore unique in its stubborn and rigorous definition of all genome edited crops as GMOs.

Non-specific or off-target effects in genome-edited crops can now be minimized or avoided altogether thanks to advances in the technology which improve target specificity substantially (Shan et al. 2015; Baysal et al. 2016; Sánchez-León et al. 2018; Macovei et al. 2018; Pérez et al. 2019). Interestingly, off-target effects which are widespread in crops developed through mutation breeding have not raised concerns from regulators, perhaps because none of these have caused serious harm in the 70-year history of mutation breeding. It is clear that targeted gene editing is the plant breeding technology with the least off-target mutations because conventional mutation breeding induces probably more than 99% of off-target mutations, and any other "natural" mutation is per se a non-target mutation, and can only in retrospect be defined as target or off-target (i.e. kept in the breeding program or eliminated), once a target is identified and followed up in precision breeding.

Case studies

Okanogan Specialty Fruits, Inc.: Arctic® apple (GD743/GS784/NF874)

The transgenic Arctic® apple (*Malus* × *domestica*) was developed by Okanogan Specialty Fruits, Inc. (OSF) to prevent enzymatic browning of the fruit flesh following exposure to air (Carter 2012; Lewis 2017; Stowe and Dhingra 2021). Browning was identified by OSF as an important and undesirable quality-related trait in multiple interviews and surveys of growers, packers, processors and consumers (Brooks 2012, 2013). A research program to develop anti-browning approaches began in 1996 when most GMO research was still focusing on input traits such as herbicide and pest resistance (Brooks 2016). The first Arctic® apple was released in 2017 in the form of pre-packaged fruit slices, and three varieties are currently available under the Arctic brand: Golden Delicious, Granny Smith and Fuji.

The Arctic® apple is a product of *Agrobacterium*-mediated transformation and is therefore classed as a GMO (Carter 2012). Browning is prevented by reducing PPO activity in the fleshy part of the fruit, which is achieved by the expression of an RNA construct that triggers the formation of double-stranded RNA (dsRNA) and therefore induces RNA interference (RNAi), suppressing the expression of four PPO families at the post-transcriptional level. RNAi can be induced by various strategies, including the direct expression of dsRNA and the expression of antisense RNA matching the target mRNA, thus leading to the formation of dsRNA in vivo. In this case, however, the strategy is sense transgene post-transcriptional gene silencing (S-PTGS), in which a transgene-derived sense transcript recruits RNA-dependent RNA polymerase 6 (RDR6) allowing the production of dsRNA (Natsuume et al. 2014). As in all RNAi approaches, the dsRNA is processed by a Dicer-like enzyme (Bernstein et al. 2001) to form short interfering RNAs (siRNAs) 21–23 nt in length that assemble with members of the Argonaute (AGO) protein family to form an RNA-induced silencing complex (RISC) that can degrade matching mRNAs (Hammond et al. 2000).

To reduce PPO activity in the Arctic® apple, four conserved *Malus* × *domestica* PPO gene families (*PPO2*, *GPO3*, *APO5* and *pSR7*) were targeted using a single concatenated RNA, resulting in the silencing of all homologous genes in each family. The PPO suppressor transgene was expressed ubiquitously under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter (Carter 2012). The induction of RNAi reduced PPO activity in the mature fruit by 91% and 90% in the Golden Delicious and Granny Smith varieties, respectively (Carter 2012). The transgenic plants

also contain the selectable marker *nptII* used during selection and regeneration.

Del Monte Fresh Produce Company: Pinkglow™ pineapple (EF2-114)

The transgenic Pinkglow pineapple (*Ananas comosus*) was developed by Del Monte as a means to introduce a brand-defining distinct pink color based on a high carotenoid content, specifically the accumulation of lycopene at levels of up to 200 mg/g (Kim et al. 2015). Like the Arctic apple, it is a product of *Agrobacterium*-mediated transformation and is therefore classed as a GMO. The Pinkglow pineapple also features a flowering senescence trait that prevents the undesirable early flowering of pineapple plants (Thomas and Ebrahim 2003).

The pink flesh phenotype was achieved by introducing three transgenes to modify the carotenoid biosynthesis pathway. The first committed step in the pathway was enhanced by overexpressing the tangerine (*Citrus unshiu*) phytoene synthase gene (*Psy*), thus providing the precursors for lycopene synthesis. At the same time, the RNAi approach discussed above was used to suppress the expression of endogenous lycopene β-cyclase (*bLyc*) and lycopene ε-cyclase (*eLyc*), thus blocking the conversion of lycopene into downstream products such as β-carotene resulting in the accumulation of lycopene, a pink pigment (Thomas and Ebrahim 2003). Flowering senescence was achieved by suppressing the meristem-specific endogenous ACC synthase gene using RNAi technology, thus blocking an early step in the production of ethylene. Unlike most other crops, pineapple flower initiation is induced by ethylene, and suppressing ethylene synthesis can therefore avoid precocious flowering (Thomas and Ebrahim 2003).

Cornell University and University of Hawaii: SunUp/Rainbow papaya (55-1)

The SunUp and Rainbow varieties (CUH-CP551-8) of papaya (*Carica papaya*) were introduced in 1997 in Hawaii in an effort to reduce the impact of papaya ringspot virus, which was devastating the Hawaiian papaya industry (Tripathi et al. 2008). The resistant variety was created by introducing the papaya ringspot virus coat protein gene by particle bombardment, aiming to suppress virus replication using an approach known as pathogen-derived resistance (Fitch et al. 1992). Although the precise mechanism varies from case to case, pathogen-derived resistance may work in a similar manner to sense transgene RNAi as employed in the other two case studies and/or may involve direct effects caused by the excess coat protein (Kavanagh and Spillane 1995). It is now known that resistance is through a mechanism involving RNAi (Tennant et al. 2001). To obtain the virus-resistant plants, papaya cultivars Sunset and Kapoho were engineered with the coat protein gene (derived from the papaya ringspot virus HA 5-1 strain) and a resulting female line (55-1) with complete resistance was crossed with papaya cultivar Sunrise. The progeny were self-pollinated to generate homozygous lines of the variety SunUp (Ferreira et al. 2002). The SunUp variety was crossed with the cultivar Kapoho to produce the F1 hybrid Rainbow. The transgenic plants also contain the selectable marker *nptII* and the visual marker *gusA*—these are used during selection and regeneration but do not play any role in the product.

Discussion

V.1 Alternative approaches for the Arctic apple

The low PPO activity in the Arctic apple could conceivably be achieved in principle using conventional breeding approaches, making it exempt from all forms of GMO regulation, but the process would be long and laborious due to a large number of target genes. A close approximation could potentially be achieved by crossing an elite variety with a naturally occurring variety with minimal PPO expression such as the 'Opal' apple (Apples 2020). However, many rounds of back crossing and successive seedling screening would be needed to fix all PPO-related genes. Bottom line, if the goal is to take an apple variety that is already popular with consumers, and add just one trait to it, conventional breeding is not an option. Apple seeds can be exposed to physical or chemical mutagens at appropriate doses to induce multisite mutations, but this carries the risk of collateral effects on genes affecting growth and fertility (Ferree and Warrington 2003). Transposon mutagenesis could be used as an alternative approach (Munoz-Lopez and Garcia-Perez 2010). However, in all mutation-based methods, the major challenge is not the acquisition of mutations in PPO genes but the need to combine many different mutations in the same line to reduce overall PPO activity effectively (Han et al. 2017).

Polyploidy and interspecific hybridization are both possible in apple. For example, the Jonagold, Gravenstein and Roxbury Russet varieties are all triploid ($3x = 51$) and are occasionally used in breeding programs given their propensity to form larger fruit (Spengler 2019). The induction of polyploidy in apple can be achieved by exposing leaf explants to colchicine (Podwyszyńska et al. 2017). However, the problem is that polyploidy typically favors traits that require gene expression rather than suppression because it increases the number of gene copies in each cell and thus the amount of mRNA. Interspecific hybridization with wild *Malus* species has been used to select for disease resistance (Pereira-Lorenzo et al. 2018) and this approach could be

useful if a wild variety with minimal PPO activity were identified, but even then the introgression of alleles representing multiple loci would be a laborious process.

Inevitably, given the nature of the trait and the number of underlying genes, the only feasible alternative to transgene-based RNAi is genome editing to inactivate the PPO genes directly. This approach is entirely feasible, especially using the CRISPR/Cas9 system which has proven effective for the simultaneous knockout of multiple members of the same gene family using a single conserved gRNA as well as multiple genes representing different gene families using two or more gRNAs (Armario Najera et al. 2019). This approach might also improve on the properties of the current Arctic apple varieties because the PPO genes would be completely inactivated rather than suppressed. Under current and proposed regulatory frameworks, a genome-edited version of the Arctic apple would still be regulated in the US, at least to the point of needing a regulatory status review, because > 1 gene would need to be edited. However, given the new USDA rules, if the same RNA construct were to be used to trigger RNAi in new events in other apple varieties, these would no longer be regulated.

Alternative approaches for the Pinkglow™ pineapple

Unlike the Arctic apple and no natural cultivar, the Pinkglow pineapple is a transgenic variety that carries an overexpressed foreign gene (*Psy* gene from tangerine *Citrus unshiu*) in addition to suppressing three endogenous genes by RNAi. Transgene overexpression can be impossible to replicate by conventional breeding if the transgene is from a species that cannot be hybridized with the breeding target, in this case pineapple. However, the *Psy* gene represents the first committed step of the carotenoid biosynthesis pathway which is present in some form in most plants, so it would not be inconceivable to find a pineapple variety that naturally expresses the endogenous *Psy* gene at high levels in the fruit, as well as naturally featuring a low-level expression of *b-Lyc* and *e-Lyc* to allow the accumulation of lycopene. Lines with low levels of ACC synthase could similarly be identified to prevent precocious flowering. Although pineapple is the only edible bromeliaceae and no cultivar is known with the required Pinkglow and/or senescence phenotypes, mutagenesis could be used to introduce such traits as it was previously implemented to reduce the number of spines (Joy and Anjana 2015). The recreation of the Pinkglow pineapple by mutagenesis would be simpler in principle than the recreation of the Arctic apple because only four mutations would be required, although one of these would need to be a promoter mutation that triggered the upregulation of the endogenous *Psy* gene. In practice, however, it would be much more difficult due to the more laborious breeding process. Furthermore, pineapple is self-incompatible and most cultivars are diploid, with only a single triploid cultivar found in Brazil (Joy and Anjana 2015). Ploidy manipulation, therefore, cannot be used to recreate the Pinkglow pineapple, and interspecific hybridization is also an unsuitable approach because of the limited genetic diversity among compatible species.

As discussed for the Arctic apple, the only feasible process to recreate the Pinkglow pineapple is CRISPR/Cas9 genome editing, which could simultaneously knock out the *bLyc*, *eLyc* and *acc* genes (Li et al. 2018b). Given that RNAi is not 100% effective, the complete knockout of *bLyc* and *eLyc* might be sufficient to recreate the Pinkglow pineapple phenotype without overexpressing *Psy* because there would be no leaky expression of the corresponding enzymes and therefore a complete metabolic block after lycopene. Alternatively, genome editing could be used to strengthen the endogenous *Psy* promoter or knock-in a strong pineapple promoter upstream of the endogenous *Psy* gene, which in many jurisdictions would still allow the resulting line to be classified as natural rather than transgenic because no foreign DNA need be involved. Alternatively, there are now several genome editing strategies that can be used to introduce random mutations scanning along the sequences upstream of target genes in order to develop allelic series based on a range of promoter configurations, and such approaches could be used to develop variants with stronger *Psy* promoters (Yan et al. 2016; Rodríguez-Leal et al. 2017).

Alternative approaches for the SunUp/Rainbow papaya

It is likely that papaya ringspot virus-resistant papaya varieties could be generated by conventional breeding given the documented existence of resistance genes in the papaya gene pool, but the genes identified thus far are QTLs that confer partial resistance or tolerance, and it would be difficult to replicate the complete and durable resistance achieved by expressing the viral coat protein gene (Yu et al. 2009; Gonsalves et al. 2008) or using replicase genes to the same effect (Kung et al. 2012; Brunetti et al. 2001; Ehrenfeld et al. 2004; Hashmi et al. 2011; Vadlamudi et al. 2020) The time and effort needed to achieve resistance, even if additional genetic diversity was introduced by mutagenesis, would not make this approach economically feasible (Teixeira et al. 2007). Polyploidy can be induced in papaya, although this approach has not yet been used in breeding programs (Clarindo et al. 2008). Furthermore, related species such as *C. pubescens, C. stipulata* and *C. quercifolia* possess compete for resistance to papaya ringspot virus, but again the time and effort required for hybridization and backcrossing to introgress the corresponding traits into elite papaya lines would be difficult to justify (Azad et al. 2014). Resistant papaya lines

have already been produced by intergeneric hybridization (*C. papaya* × *C. pubescens*) but only female individuals were recovered (O'Brien and Drew 2009).

In the case of SunUp/Rainbow papaya, genome editing does not provide a shortcut to the recreation of the variety with a lower regulatory burden. Although genome editing may, in time, be used to inactivate multiple loci that confer susceptibility to papaya ringspot virus, the use of genome editing to introduce the virus coat protein gene does not provide any advantage over the standard transgene delivery method except that it would be possible to preselect the transgene integration site and, potentially, avoid the use of a selectable marker (after segregation). Due to the presence of a foreign gene in the papaya genome, the SunUp/Rainbow variety would continue to be classed as a GMO in all jurisdictions. However, in the US, a new papaya made with a coat protein gene that triggers silencing would no longer be regulated by the USDA, but the EPA would still regulate it as a pesticide.

Conclusions

We have discussed the different breeding technologies that can be used to improve fruit crops and how these technologies fit within the regulatory frameworks that cover different parts of the world. Although these frameworks may be based on either the product (USA/Canada "substantial equivalence" model) or the process (EU "precautionary principle" model), even the process-based regulations generally make a distinction between genome-edited varieties that replicate natural mutations and those incorporating foreign genetic material, the EU being the exception. The dogmatic position of the EU, adopting a stance against the rest of the world and apparently also against common sense, will make international trade more difficult in the future as genome-edited crops become more prevalent. For two of the three case studies we examined, genome editing could accurately replicate the phenotype generated in these transgenic plants but would avoid the introduction of any foreign DNA. Furthermore, if we take the pertinent example of DNA-free genome editing using ribonucleoprotein particles, it is clear from a scientific perspective that the mutagen, in this case the Cas9 ribonucleoprotein, is no different than a burst of gamma rays or a dose of EMS, in that it is transiently active in the plant cells and is not inherited. However, it is very different in that it can be used to mutate one gene in a specific manner, in contrast to gamma rays and EMS, which are scattergun approaches requiring the generation of large mutant populations to identify rare desirable events. The EU position is that bombarding seeds with gamma rays and hoping for the best is a superior approach to creating a precision genetic change with a molecular scalpel. Similarly, it is absurd that techniques such as interspecific hybridization, which mix all the genes from one plant with all the genes from another, effectively creating the 'ultimate transgenic plant', should be regarded as somehow more natural than the precise introduction of a single gene from a resistant wild species into a related crop variety using a clean genome editing approach that leaves no other genetic footprints. One must also pay attention to the relative time investment required to develop new varieties especially given the pressures of a changing socio-economic and climate landscape. It is time to reconsider some of the regulatory practices that have blocked timely development of cultivars. The case studies show that replacing transgenic varieties with gene-edited alternatives would satisfy the regulatory agencies in most jurisdictions at least under certain conditions (e.g. in the US if only one edit is involved and the plant has not been modified for pest or disease resistance) but not the EU, which continues to enact its legislation with regard to political expediency and activism rather than rigorous scientific evidence.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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