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SPECIAL ISSUE ON GENOME EDITING

Gene editing in tree and clonal crops: progress and challenges

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Abstract
Because of the limitations inherent in conventional breeding of trees and clonally propagated crops, gene editing is of great interest. Dozens of published papers attest to the high efficiency of CRISPR-based systems in clonal crops and trees. The opportunity for "clean" edits is expected to avoid or reduce regulatory burdens in many countries and may improve market acceptance. To date, however, nearly all studies in trees and clonal crops retained all of the gene editing machinery in the genome. Despite high gene editing efficiency, technical and regulatory obstacles are likely to greatly limit progress toward commercial use. Technical obstacles include difficult and slow transformation and regeneration, delayed onset of flowering or clonal systems that make sexual segregation of CRISPR-associated genes difficult, inefficient excision systems to enable removal of functional (protein- or RNA-encoding) transgenic DNA, and narrow host range or limited gene-payload viral systems for efficient transient editing. Regulatory obstacles include those such as in the EU where gene-edited plants are regulated like GMO crops, and the many forms of method-based systems that regulate stringently based on the method vs. product novelty and thus are largely applied to each insertion event. Other major obstacles include the provisions of the Cartagena Protocol with respect to international trade and the need for compliance with the National Environmental Policy Act in the USA. The USDA SECURE act has taken a major step toward a more science- and risk-based—vs. method and insertion event based—system, but much further regulatory and legal innovation is needed in the USA and beyond.

Keywords Genome editing, · Forest Biotechnology, · Clonal propagation

Introduction

Trees and clonally propagated plants represent a large portion of cultivated plant species. They are used for food, fiber, and fuel, and include some of the best-known crops in the world, including banana, apple, citrus, grape, stone fruits, nut trees, sugar cane, potato, mint, and numerous species of forest trees used for wood production. Many of these crops have ancient associations with agriculture, and some clones have had hundreds to thousands of years of continual propagation and cultivation, as is evidenced by recent genomic characterizations (Duan *et al.* 2017; Wu *et al.* 2018; Vondras *et al.* 2019). For clonally propagated plants, perpetual retainment of genotype-unique traits and heterosis is an important trait common to many cultivars. Coupled with delayed onset of reproduction, low tolerance of inbreeding, and natural sterility, many of these species are slow and difficult to breed. In addition, oligogenic and recessive traits of high value, such as those affecting product quality and pest resistance, are nearly impossible to breed for when they are rare in gene pools. Thus, by enabling the efficient production of loss of function traits, gene editing provides a powerful new tool that was essentially unavailable in tree and clonal crops.

The same traits that make sexual breeding difficult in tree and clonal crops, however, make gene editing and genetic engineering challenging. It is difficult to introgress new edits or transgenes into diverse lines and also difficult to remove editing machinery from the genome by sexual segregation, as is common in annual crops. In addition, many of these species are also notoriously difficult to genetically modify and regenerate (Birch 1997; Busov *et al.* 2005). Even in species where genetic transformation has been accomplished, such as poplars and eucalypts, most genotypes remain extremely difficult to transform (Nagle *et al.* 2018). Thus, new technologies that can speed genetic modification and gene editing, or reduce obstacles to their regulatory acceptance, are crucial.

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There are several classes of technologies that can promote the production and acceptance of gene-edited trees and clonal crops. Technologies such as the use of developmental regulators (DRs) for example *GROWTH REGULATORY FACTOR 4* (Debernardi *et al.* 2020), among many others, to aid regeneration of genetically modified cells have been reviewed elsewhere and will not be covered here (Nagle *et al.* 2018). However, there have been very few reports of their successful use in tree and clonal crops (Florez *et al.* 2015; Brand *et al.* 2019; Maher *et al.* 2020), thus appear to be important technologies for further development. The use of DRs is likely to require transient expression or excision from the genome (Hoerster *et al.* 2020), in common with the desire to avoid permanent insertion of gene editing machinery from genomes (to avoid off-target effects and ease regulatory or market approval). Thus, most of the technologies we discuss below can have benefits beyond facilitating the production and acceptance of gene-edited varieties. We first briefly review recent developments in CRISPR technology with special relevance to tree and clonal crops, then discuss options for transient editing, transgene excision, and accelerated flowering to enable segregation of gene editing components away where breeding methods allow it.

CRISPR/Cas Techniques: a Rapidly Evolving Toolset for Plant Genetic Modification

The previous decade has seen the rapid development of reliable and versatile molecular tools for the targeted modification of endogenous plant genomes, which has been a broad and rapid departure from mainly transgene-based tools such as overexpression, silencing, misexpression, and introduction of novel traits, which together dominated plant molecular biology throughout the 1990s and 2000s. While a number of effective site-directed nucleases such as TAL-like effector nucleases (TALENs) and designer zinc finger nucleases (ZFNs) have been successfully used in plants, in recent years, CRISPR/Cas9 systems have become most common (reviewed in Razzaq *et al.* 2019) Many recent variants of the original Cas9 system from *Streptococcus pyogenes* (spCas9) have expanded the toolbox of the types of modifications that can be accomplished, highlighting the versatility of Cas protein systems.

The main functional attributes of CRISPR systems are the capacity for mutations in target genes, directed single base changes, and directed sequence modifications (termed homology-directed replacement: HDR) (reviewed in Chen *et al.* 2019). Directed mutations lead to loss of function through frame-shift mutations or large deletions, which can be done in multiple genes simultaneously at high efficiency. There are many examples of such applications in trees and other clonal crops (summarized in Table 1). By fusion of catalytically dead Cas9 to natural or synthetic cytosine or adenine deaminases, targeted changes to individual bases can be accomplished without the use of a donor template (reviewed in Bharat *et al.*. 2020). Traits such as herbicide resistance traits have been modified using this method (Li *et al.* 2018) and were recently demonstrated in sugarcane (a clonal crop) (Oz *et al.* 2017). HDR was also recently shown in cassava to tag an endogenous gene with GFP to monitor expression levels (Veley *et al.* 2021); however, to our knowledge, no trait-focused non-herbicide-resistant traits have been shown in a tree or clonal crop using HDR. HDR in comparison is far less efficient than direct mutations or base editing, and as discussed below raises larger regulatory concerns as they are generally treated as regular GMOs.

Because rates of HDR are low compared with directed mutation, several methods have been adapted to improve efficiencies. These include the use of viral replicons that increase donor template copy number (Čermák *et al.* 2015). A second means to improve rates of directed sequence replacement is to increase the temperature during the early stages of transformation, as the efficiency of many Cas proteins seems to be tailored toward warmer temperatures than are commonly found in plant *in vitro* culture (22–25°C) (Malzahn *et al.* 2019; Milner *et al.* 2020). Still, many reports from stable integration of Cas9 and gRNAs in several species report 10% or lower rates of HDR incorporation of a donor template. Cas9 modified fusions to proteins such as virD2 may further increase these rates in addition to other potential strategies (Begemann *et al.*, 2017; Čermák *et al.*, 2017; Ali *et al.*, 2020; Dong *et al.*, 2020). Another technological improvement useful for directed sequence replacement is "prime" editing (Anzalone *et al.* 2019). It does not require a DNA template nor homology-driven recombination and thus should be more efficient than HDR. So far, this has been demonstrated in rice, wheat, and potato but at fairly low frequencies of 10–20% (Lin *et al.* 2020; Veillet *et al.* 2020b). Though inefficient at present, these approaches greatly increase the types of genomic modifications available, such as insertion of promoter elements to change endogenous expression and directing transgenes into "safe harbors" (*i.e.*, well-characterized zones of DNA unlikely to be affected by transgene silencing or affecting the function of native genes).

Means to Produce Edited Trees/Clonal Crops Without Permanent Cas9/gRNA Integration

The dominant method for the development of CRISPR/Cas9-mediated edits in the genomes of trees and clonally propagated crops is through stable *Agrobacterium*-mediated transformation (see Table 1 for recent trends). Generally, this would involve delivery of Cas9, gRNAs, and plant resistance marker genes in a single T-DNA. While it is routine in many

Table 1 Gene editing in trees and clonal crops 2017–present.

Genus	Editing strategy (stable (S), transient (T), virus (V), (# studies))	Traits for improvement
Gymnosperms		
Pinus radiata	S(1)	Cell wall modification
Dicots		
Hevea T(1)	*Hevea* T(1), Cas9-RNPs	Flowering time
Populus	S(18)	Flowering traits, sterility, branching form, sex, determination, lignin contents, photoperiodism, anthocyanin biosynthesis, defense biochemistry
Malus	S(4),T(2), recombinase excision, transient *Agrobacterium*, Cas9-RNPs	Disease resistance, herbicide resistance
Pyrus	S(1)	Flowering time
Parasponia	S(1)	Nodulation, hormone signaling
Eucalyptus	S(2)	Wood quality, sterility
Coffea	S(1)	- *PDS* editing only
Theobroma	S(1)	Disease resistance
Camellia	S(1)	Caffeine biosynthesis
Citrus	S(8), T(1), Cas9-RNPs	Disease resistance
Actinidia	S(4)	Sex determination
Vaccinium	S(1)	Flowering traits
Humulus	S(1)	- *PDS* editing only
Vitis	S(6), T(1), Cas9-RNPs, recombinase excision	Disease resistance
Manihot	S(5)	Starch content, disease resistance
Solanum	S(11), T(3), transient *Agrobacterium*, Cas9-RNPs	Starch content, disease resistance, herbicide resistance, self-incompatibility
Ipomoea	S(1)	Starch content
Fragraria	S(9)	Hormone signaling, fruit color, flowering, fruit sugar contents
Monocots		
Musa	S(7), T(1), Cas9-RNPs	Dwarfism, disease resistance, beta-carotene content
Saccharum	S(2)	Herbicide resistance, lignin content

Sources supporting the table row entries are listed in Table S1, and include the following: *Pinus* (Poovaiah *et al.* 2020), *Hevea* (Fan *et al.* 2020), *Populus* (Wan *et al.* 2017; Wang *et al.* 2017; Yang *et al.* 2017; Elorriaga *et al.* 2018; Fan *et al.* 2018; Muhr *et al.* 2018; Ramos-Sánchez *et al.* 2019; An *et al.* 2020; De Meester *et al.* 2020; Della Maggiora 2020; Ding *et al.* 2020; Fellenberg *et al.* 2020; Movahedi *et al.* 2020; Müller *et al.* 2020; Qin *et al.* 2020; Tsai *et al.* 2020; Wang *et al.* 2020; Azeez and Busov 2021), *Malus* (Osakabe *et al.* 2018; Charrier *et al.* 2019; Dalla Costa *et al.* 2020; Pompili *et al.* 2020; Zhou *et al.* 2020a), *Pyrus* (Charrier *et al.* 2019), *Citrus* (Jia *et al.* 2017b; Jia *et al.* 2017a; Peng *et al.* 2017; Jia *et al.* 2019; Zhu *et al.* 2019; Zou *et al.* 2019; Dutt *et al.* 2020; Huang *et al.* 2020; Jia and Wang 2020; Omori *et al.* 2021), *Parasponia* (van Zeijl *et al.* 2018), *Coffea* (Breitler *et al.* 2018), *Theobroma* (Fister *et al.* 2018), *Camellia* (Ma *et al.* 2021), *Actinidia* (Wang *et al.* 2018b; Akagi *et al.* 2019; Varkonyi-Gasic *et al.* 2019; Varkonyi-Gasic *et al.* 2021), *Humulus* (Awasthi *et al.* 2021), *Vitis* (Nakajima *et al.* 2017; Osakabe *et al.* 2018; Wang *et al.* 2018a; Ren *et al.* 2019; Dalla Costa *et al.* 2020; Li *et al.* 2020a; Sunitha and Rock 2020; Awasthi *et al.* 2021), *Manihot* (Odipio *et al.* 2017; Gomez *et al.* 2019; Chatukuta and Rey 2020; Li *et al.* 2020b; Veley *et al.* 2021), *Solanum* (Andersson *et al.* 2018; Hummel *et al.* 2018; Kusano *et al.* 2018; Nakayasu *et al.* 2018; Enciso-Rodriguez *et al.* 2019; Johansen *et al.* 2019; Osmani *et al.* 2019; Tuncel *et al.* 2019; Veillet *et al.* 2019; Yasumoto *et al.* 2019; Bánfalvi *et al.* 2020; González *et al.* 2020; Sevestre *et al.* 2020; Veillet *et al.* 2020b; Veillet *et al.* 2020a), *Ipomoea* (Wang *et al.* 2019), Vaccinium (Omori *et al.* 2021), *Fragraria* (Xing *et al.* 2018; Zhou *et al.* 2018; Feng *et al.* 2019; Martín-Pizarro *et al.* 2019; Wilson *et al.* 2019; Alvin 2020; Gao *et al.* 2020; Xing *et al.* 2020; Zhou *et al.* 2020b), *Eucalyptus* (Dai *et al.* 2020), *Musa* (Kaur *et al.* 2018; Naim *et al.* 2018; Tripathi *et al.* 2019; Hu *et al.* 2020; Kaur *et al.* 2020; Ntui *et al.* 2020; Shao *et al.* 2020; Wu *et al.* 2020), *Saccharum* (Oz *et al.* 2017; Kannan *et al.* 2018)

herbaceous species to self-pollinate such transgenic plants and then screen progeny for a combination of desired edits and absence of the CRISPR-related genes, it is very difficult to do in trees and clonally propagated crops (Nadakuduti *et al.* 2018). This is often due to the time delay to onset of flowering (especially in trees), intolerance of inbreeding, sterility of hybrid or polyploid varieties, and loss of clonal integrity due to sexual segregation. If the Cas9 and gRNA genes must be removed from edited lines due to regulatory needs or biological concerns (discussed below), methods for editing without integrated transgenes, or technologies for removing integrated transgenes, will be required. In the following sections, we outline the general methods that are available and the subject of active research (class I–IV, Fig. 1). We do not consider the case for retention of gene editing machinery in commercial plants, although evidence for extremely low rates of off-target

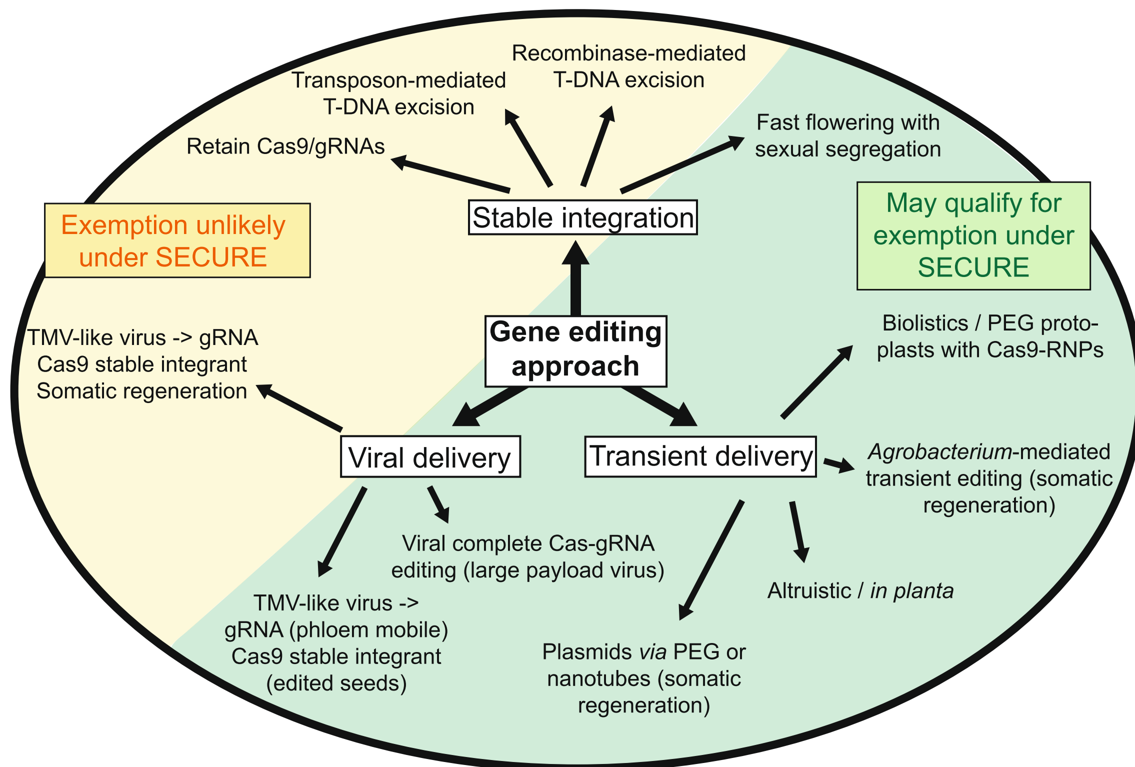

Figure 1. Methods for gene editing product development in trees and clonally propagated crops and anticipated regulatory outcomes in the USA under USDA SECURE. Gene editing via CRISPR/Cas9 can be accomplished through several approaches in trees and clonally propagated crops. These involve stable integration of an editing transgene in the genome (top arrow), viral delivery methods (left arrow), and transient delivery systems (right arrow). Under SECURE, methods detailed in the *green* region are likely to be exempt from regulation so long as the trait meets eligibility criteria and only single and simple edits are made that mimic potential natural variants. Methods shown in the *yellow* highlighted are likely to require a Regulatory Status Review (RSR) similar to that for other transgenic traits. Stable integration methods are the most common at present; in clonally propagated crops, somatic transgene removal systems mediated by site-specific recombinases or transposable elements have been demonstrated, though to date only recombinases have been used in conjunction with gene editing. If sexual segregation is possible, accelerated flowering systems can be used to remove stably integrated editing transgenes. Transient delivery methods can be accomplished by various DNA delivery systems (*Agrobacterium*, plasmids by physical delivery) or DNA-free methods (Cas9/-RNPs). These methods must be efficient enough to produce edited non-chimeric shoots, or protoplast regeneration from single cells, at a rate adequate to address breeding program demands. Viral-only delivery methods could be used if the viral genome has enough capacity for a Cas enzyme (rhabdoviruses, Potato virus X) and the gRNAs. If viral systems are used only to deliver gRNAs during systemic infection, mixed systems with stable Cas9 lines would be required.

mutation in plants (Young *et al.* 2019), reviewed in Graham *et al.* (2020) and Modrzejewski *et al.* (2020), and recent evidence for a virtual absence of vegetative consequences of continued CRISPR/Cas9 expression in eucalypts (Elorriaga *et al.* 2021), provides support for this option. If CRISPR machinery is retained, there may be regulatory or market concerns over potential gene drives affecting domesticated or wild/feral populations. However, because of the long generation time or low fertility/sterility of many tree and clonal crops, we expect that at least the biological concerns over possible gene drive to be negligible.

Class I. Stable Integration of Gene Editing Components Followed by Removal of Functional Components *Via* Gene Excision The removal of parts of transgenes has been a key component of transgenic research in plants for several decades, where focus has been on removal of antibiotic or herbicide resistance markers for regulatory acceptance (reviewed in Tuteja *et al.* 2012). Another benefit of marker removal is so that elite-generated insertion events can be stacked with further transgenic traits using the same selectable marker. Generally, marker removal from integrated transgenes by recombination is not possible even in annuals with high fecundity because of the tight linkage between the desired trait and the selectable marker in the same transgene. This necessitated methods where parts of the transgene could be selectively excised after the initial transformation process, and these usually focused on the somatic removal of selectable marker genes using recombinases such as Cre from P1 bacteriophage and FLP from yeast (Kilby *et al.* 1995; Wang *et al.* 2005; Hu *et al.* 2008; Kim *et al.* 2012), although more exotic recombinases have been used such as R, ParA, and CinH (Schaart *et al.* 2004; Shao *et al.* 2017). In these strategies, recognition sites for the recombinases are included at flanking

regions where parts of transgenes are desired to be deleted (the selectable marker and usually including the recombinase gene). These could be expressed from inducible promoters (*e.g.*, heat shock or cold shock) or developmental promoters (*e.g.*, meristematic or reproductive; Fig. 2(*B*)) to decrease the rates of premature marker removal during the initial phases of transformation, but allow for the excision to occur once integrated transgenic events are isolated or potentially characterized (Fig. 2). These methods have been adapted to the removal of CRISPR-related genes in two recent studies in apple and grape where the FLP recombinase was expressed from a heat shock promoter and flanking recognition sites were placed near the T-DNA borders (Pompili *et al.* 2020). After transgenic lines were isolated and identified *in vitro* for traits predicted to confer blight resistance, the CRISPR-related genes were excised using heat shock. The method was effective for removing the editing transgene but required recovery and further regeneration *in vitro* of the material due to the prolonged, high temperature required for adequate excision. A second study in apple and grape using the same approach and a novel second method using CRISPR/Cas9-mediated deletion of the transgene showed rapid but incomplete excision after 3 h of heat shock with FLP recombinase and significantly less excision using Cas9 (and additionally, issues with CRISPR target sites being irreversibly mutated to prevent excision) (Dalla Costa *et al.* 2020). When taking into account marker removal studies, recombinase excision approaches have been validated in several tree and clonally propagated

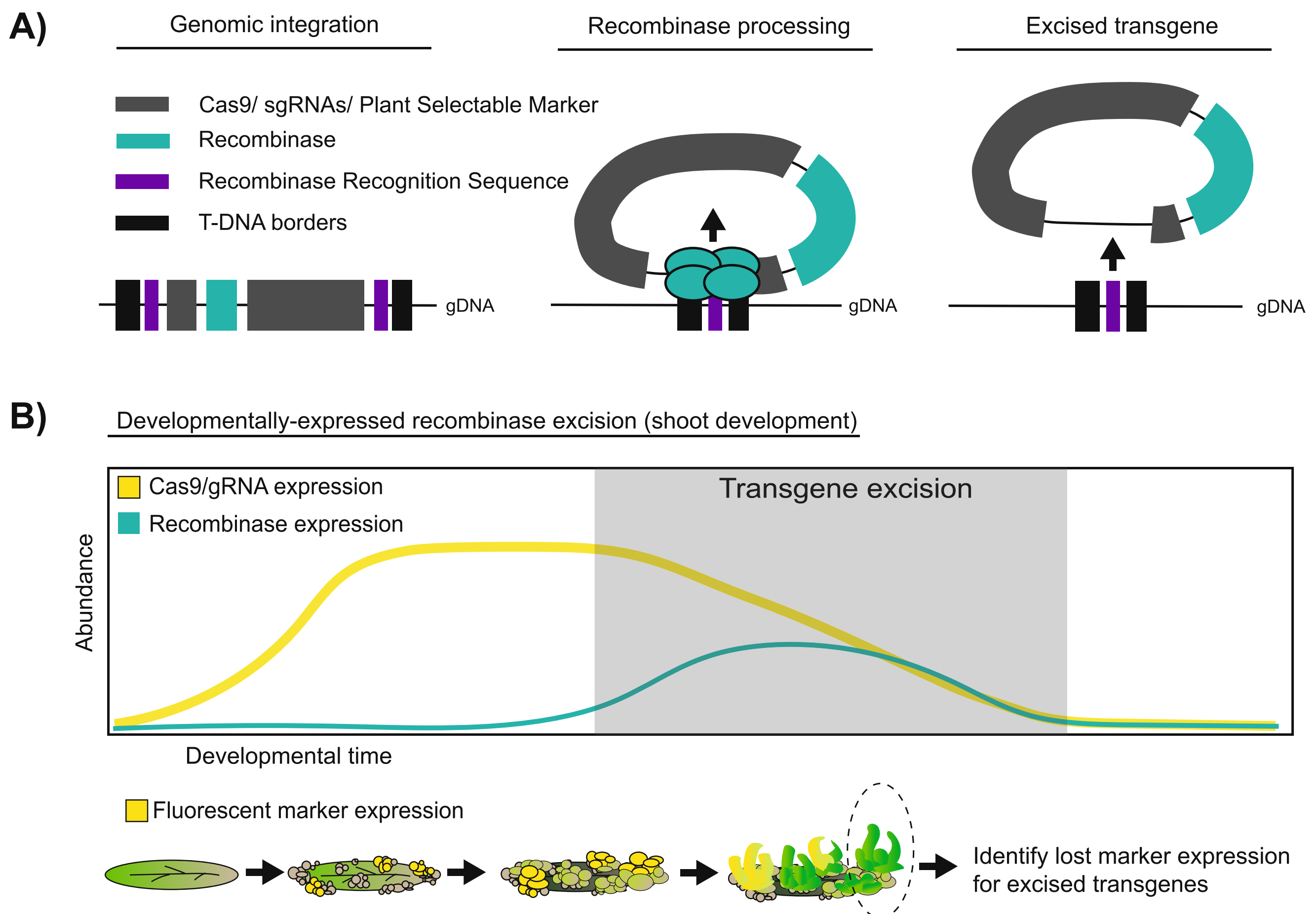

Figure 2. Method for coupling stable transgene integration followed by controlled recombinase-mediated excision to generate edited clonal plants with minimal remaining inserted DNA. (*A*) Transgenes delivered by *Agrobacterium* containing all the elements required for gene editing and transgene removal are stably integrated into the genome. When recombinases are expressed, they bind recognition sequences flanking the T-DNA region and excise the transgene through circularization and subsequent degradation. (*B*) Though many schemes for recombinase control after gene editing are possible (heat shock, reproductive development-triggered expression, chemical induction), developmental control of recombinase expression in shoot meristems would allow for the production of edited shoots with eliminated transgenes in a single step when used with common organogenic *in vitro* transformation systems. Shoots without fluorescent marker genes could be retained and analyzed for desired edits. For species and genotypes with low transformation rates, stable integration and recombinase-mediated excision methods are likely to be beneficial over transient or DNA-free methods, due to selection and positive evidence of transgenic insertion. The main challenge is the identification of developmental promoters and associated insulator elements that give desired and reliable expression levels in transgenic target tissues; a combination of developmental and environmentally or chemically induced expression may be needed for such a system to work reliably.

species, including poplars, strawberry, potato, and apple, highlighting their versatility (Schaart *et al.* 2004; Kondrák *et al.* 2006; Fladung *et al.* 2010; Fladung and Becker 2010; Timerbaev *et al.* 2019). However, improvement of the typically low excision rates seen may require mitigation of target site methylation (Liu et al. 2021).

A drawback of this approach is that a small residual "footprint" from the original T-DNA insertion will still be present in the genome. If the original insertion event landed within the coding sequence of an existing gene, the function of that gene will likely still be affected after the transgene is excised. If a particular insertion event has multiple T-DNA integrations, the complete excision of all transgenes may be difficult to accomplish, and their resolution may give rise to complex rearrangements or deletions of large sections of native DNA (when the T-DNAs and associated recognition sites are located in close proximity on the same chromosome) (Wang *et al.* 2011). Thus, as discussed in depth below, under extant regulatory regimes, the plants, though lacking any transgenically derived functional (protein or RNA encoding) transgenes, may face similar regulatory hurdles to conventional GMOs (Fig. 1)—though of course chromosomal rearrangements are a common feature of conventional breeding that employs wide crosses, somaclonal variation, or radiation or chemical mutagenesis (Bradford *et al.* 2005; Fossi *et al.* 2019; Jo and Kim 2019).

Transposable elements can serve a similar function to recombinases. In this strategy, the transgene could be delivered with a functional transposase gene and recognition sites at the borders of the transgene to direct the transposase to excise. Over time, the integrated transgene might be excised completely and not be integrated elsewhere in the genome. Because the maize *Ac* transposon is very well characterized and functional in monocots and many dicot plants, several studies have used the *Ac* transposase and demonstrated its function in tobacco and poplar for the removal of transgenes (Ebinuma *et al.* 1997). Recent developments in animal transformation systems have developed excision only transposase enzymes such as *piggyBac*, which could be adapted to plant excision systems, but have only been demonstrated in a single recent study in rice (Nishizawa-Yokoi and Toki 2021). In demonstrated examples in plants, this strategy has a low frequency of excision and takes several months or longer to obtain fully non-chimeric, excised events. These drawbacks explain the increased focus on recombinase excision systems both for marker removal and editing transgene removal in trees and clonal crops.

Class II. Transient DNA Delivery (No Integration) Stable integration of CRISPR-related genes in the plant genome has been repeatedly shown to give rise to non-chimeric, edited events at high frequency (Table 1). However, as discussed above, the complete removal of gene editing components without sexual reproduction is problematic in trees and many clonal crops. One obvious solution is to avoid integrating the transgene in the first place—relying on transient expression of editing agents. There are several methods that have been demonstrated or proposed to accomplish this, and they all share similar drawbacks when compared to editing by stable transgene integration. These include the difficulties of obtaining non-chimeric plants without strong selection using antibiotic or herbicide resistance genes, and overall low rates of editing in regenerated plants (Chen *et al.* 2018). The latter is a serious obstacle for species with low transformation rates, as normally tens to many hundreds of treated plants must be screened to find a small number of homozygous knockout edited plants (Bánfalvi *et al.* 2020). However, because the gene-edited plants generated using these approaches will be exempt in many countries (discussed below), there has been a flurry of interest in their development.

The first such technique is to borrow what is commonplace in animal CRISPR/Cas9 editing systems—*i.e.*, simply to provide the Cas enzyme and associated guide RNAs as a pre-packaged ribonucleic protein complex (RNP) without any DNA (unless a donor template is required for HDR) (Chen *et al.* 2016). Using this approach requires a physical delivery method for the Cas9/sgRNA RNPs, similar to how fertilized zebrafish or mouse embryos are often injected (Hoshijima *et al.* 2019). In plants, the most common method employed has been to use polyethylene glycol (PEG) physical transformation with cultured protoplasts (which have the cell wall largely removed), then to regenerate the protoplasts into intact plantlets *in vitro* (Murovec *et al.* 2018; Wu *et al.* 2020). Generally, the use of DNA-free delivery and of single-celled source material greatly reduces the problem of chimeric regenerants, provides a high rate of editing, and eliminates the possibility of transgene integration (Metje-Sprink *et al.* 2019). The main drawbacks are that protoplast regeneration systems are very difficult to develop and reproduce in most plant species, especially trees, and protoplast regeneration methods are well known to cause a high rate of somaclonal mutation (Roest and Gilissen 1989; Bairu *et al.* 2011; Fossi *et al.* 2019). For example, when comparing protoplast and explant-based regeneration in potato, the former was found to cause a much higher rate of structural somatic mutations (Fossi *et al.* 2019). Another drawback is the frequent integration of small fragments of DNA at CRISPR target sites. In rice, a significant proportion of Cas9 RNP–derived lines contained DNA integrated at target cut sites whose origin was most likely explained by contamination from various sources of plasmid DNA (Banakar *et al.* 2019). More research in other species, and with contrasting transformation approaches, is needed to understand how widespread this problem is. A few examples of Cas9-RNP-mediated gene editing have been demonstrated in clonal crops and are discussed in greater detail below and found in Table 1.

A second method by which edited but transgene-free events can be obtained is using standard *Agrobacterium* transformation techniques but applying little or no antibiotic selection. This approach was demonstrated in tobacco (Chen *et al.* 2018); however, very low rates of successful edited and transgene-free events were obtained (0.4%). Potentially, modifications to *Agrobacterium* T-DNA function which increase infection and transient T-DNA delivery, but hinder the rate of transgene integration, may improve this approach. For example, modifications in proteins essential for T-DNA delivery such as VirD2, the protein which assists in processing and piloting the T-DNA via interaction with the T-DNA right border (RB) sequence, or plant factors involved in DNA repair may be important for reducing the rate of T-DNA integration (Mysore *et al.* 1998; van Kregten *et al.* 2009; van Kregten *et al.* 2016; Nishizawa-Yokoi *et al.* 2021).

Another option for improvement of transient editing is to have a short period of antibiotic selection when transient expression is highest. One study in potato saw no significant increase in edited, transgene-free plant recovery using this approach and was unable to recover non-chimeric edited shoots (Bánfalvi *et al.* 2020). Another study in apple succeeded in retaining transgene-free edited shoots using a visual editing marker, but at a 0.26% rate compared with all regenerated shoots (Charrier *et al.* 2019). At present, the rates of edited, transgene-free events may be too low to be useful in the many species of trees and clonally propagated crops with poor transformation rates.

Developmental genes that aid in regeneration of genetically modified cells could also improve the rate of transient editing. Regeneration aiding genes such as *WUSCHEL (WUS)* or *BABY BOOM (BBM)*, whose proteins can move between cells, can have a negative or toxic impact on cells stably transformed but a positive effect on the regeneration of surrounding cells (Lowe *et al.* 2016; Hoerster *et al.* 2020). Such "altruistic" approaches typically employ a two-vector strategy where sacrificial cells retain the developmental gene encoding T-DNA, while neighboring cells are hopefully modified but do not see developmental gene integration (Hoerster *et al.* 2020). This approach is desirable because it may increase the rate of transformation in difficult to regenerate genotypes. This type of editing was recently shown for "*in planta*" transformation in tobacco, grape, tomato, and potato with *Agrobacterium* (Maher *et al.* 2020). In this study, a pair of developmental genes (*IPT/WUS* or *BBM* or *SHOOT MERISTEMLESS* (*STM*)) together with sgRNAs (all encoded by a single T-DNA) was applied to meristematic tissues of a stable Cas9-expressing plant under growth chamber or greenhouse conditions. Although only a few seeds and shoots were analyzed, most appeared to be edited but lacking stable T-DNA integration. Although the generation of a stable Cas9 expressing founder lines was an added complication of this approach, this might not be needed (*i.e.*, Cas9 might be provided in the binary vector, as is common in gene editing studies). Additional studies to replicate this work, including in other plant species and in some of the notoriously difficult trees and clonal crops, are needed.

Class III. Viral Delivery Approaches There has been a recent increase in the use of viral delivery systems for plant gene editing. In its most common guise, plant viruses are used to systemically delivery gRNAs throughout an infected plant (Hu *et al.* 2019; Ghoshal *et al.* 2020). These could be used to accomplish gene editing in specific tissues (where viruses replicate) and thus to obtain non-chimeric edited and virus-free plants from those tissues. The major drawback of using viruses for editing, whether they be RNA or DNA viruses, is their limited genome size and thus modest gene delivery payload, if they are to maintaining the ability to move systemically throughout the plant. Widely studied RNA viruses such as Tobacco Mosaic Virus (TMV) lose the ability for systemic infection if Cas9 (~4kb protein-coding portion) is included in the viral genome (Ellison *et al.* 2020). In general, this means that mixed approaches with previously transformed Cas9-expressing plants are required to generate non-chimeric edited plants (Figs. 1 and 3).

More advanced viral editing strategies which increase gRNA mobility in reproductive tissues may expand the versatility of these systems. In contrast to the previous strategies, some plant negative-strand RNA viruses (NSVs) such as rhabdoviruses may have the requisite payload capacity to deliver both Cas9 and the gRNAs via systemic infection. Recently, the Sonchus yellow net virus (SYNV) and the Potato virus X (PVX) were shown to be capable of delivering both Cas9 and gRNAs throughout tobacco plants (Ariga *et al.* 2020; Ellison *et al.* 2020). These studies also caused successful editing of the *PHYTOENE DESATURASE (PDS)* endogene, a GFP visual marker transgene, and a few other genes of interest. Using this system, after infection and editing, virus-free materials can be produced through somatic regeneration. Although the host range of SYNV and PVX is limited, if similar viruses can be found for suitably wide taxonomic groups (family or genera) of trees and clonal crops, and adapted for use in routine gene editing, they could be powerful methods for gene editing. A great deal of research on the molecular virology of tree and clonal crops, and broadly compatible and better delivery vectors suited for them, is clearly needed.

Class IV. Accelerated Flowering to Enable Transgene Removal *Via* Sexual Segregation in Trees and Clonal Crops Accelerated flowering in trees, and associated rapid breeding, has been demonstrated in several species (Zhang *et al.* 2010; Srinivasan *et al.* 2012; Ye *et al.* 2014; Klocko *et al.* 2016), enabling sexual segregation to remove gene editing components. Of course, such strategies could not be employed on events with engineered sterility for

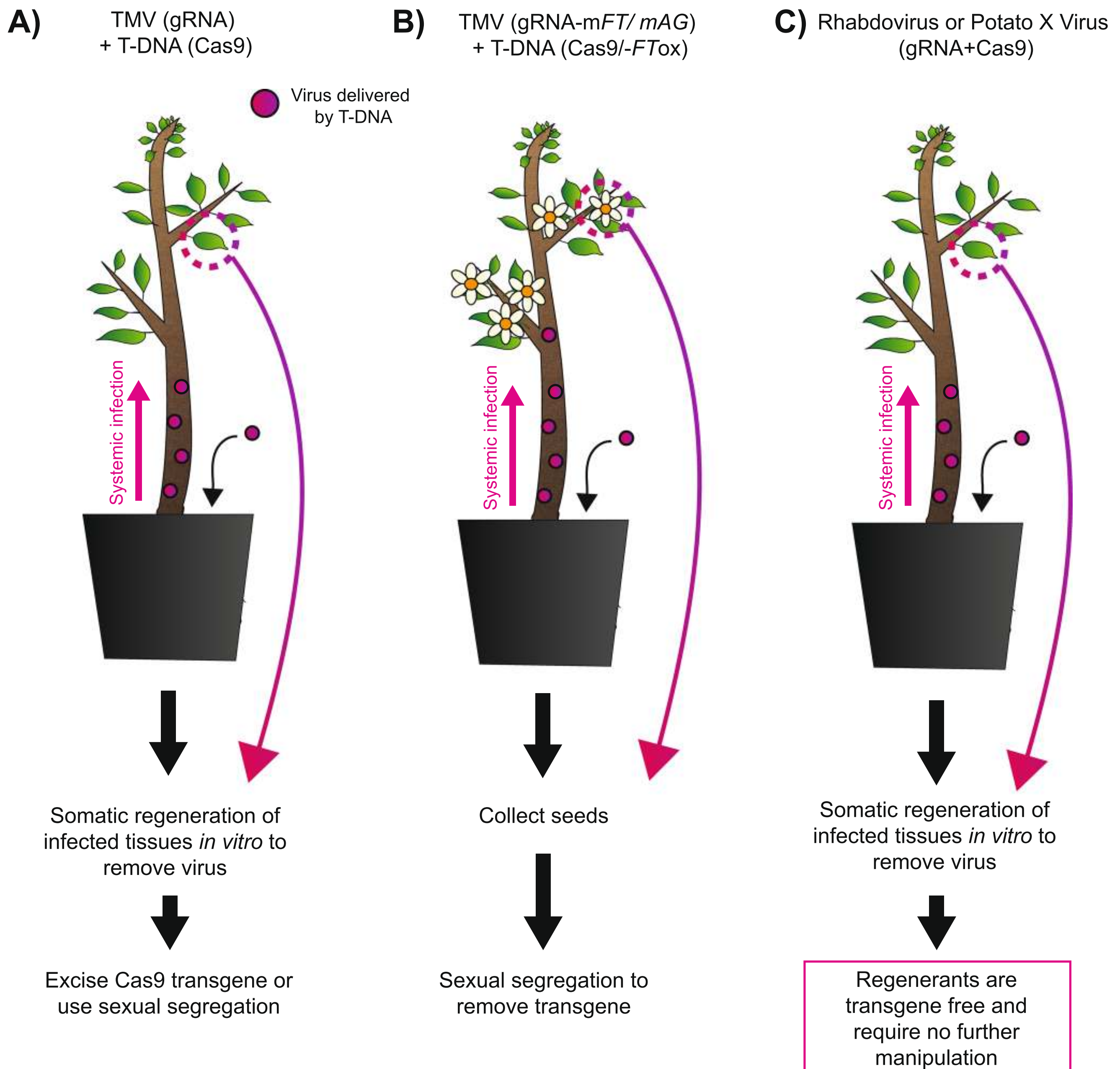

Figure 3. Viral methods for gene editing component delivery to generate gene-edited lines in trees and clonally propagated plants. (*A*) gRNAs are delivered to Cas9 transgenic plants through systemic infection of a RNA virus such as TMV that supports a small genome payload. Infection of transgenic plants already expressing Cas9 results in many edited cells in tissues bearing viral infection. Regeneration of infected leaf tissue into shoots purges the virus and frequently results in fixation for edits/non-edits due to small founder cell populations. (*B*) gRNAs are delivered by systemic infection but are also tailored with mobile RNA elements which help improve gRNA movement into reproductive tissues (Using parts of the *FT* or *AG* transcript) to areas which are normally inaccessible to viral infection. When coupled with a transgenic plant with accelerated flowering and expressing Cas9, edited seeds can be obtained, and the transgene containing Cas9 and the *FT* expression construct can be segregated out of the plant genome. (C) Negative-strand RNA rhabdoviruses with large genome sizes can be used in a manner similar to the method outlined in (*A*), however, do not require a Cas9 transgenic plant to infect, simplifying the generation of edited plants.

containment (*e.g.*, some proposed plantation forest trees) or sterile genotypes (*e.g.*, peppermint). In many cases due to the absence of transformability of desirable elite cultivars, editing will likely need to be performed in a transformable genotype, followed by successive backcrossing to the elite cultivar to obtain marketable lines. This has been demonstrated as a viable strategy in plum for the introgression of plum pox virus resistance traits (Petri *et al.* 2018), where there is a single gene being introgressed. As a result of rapid flowering, the desired introgression can be completed in less than a decade, as opposed to the multiple decades such an effort in plum would normally require.

For more complex breeding programs where there are polygenic trait targets and the need for many gene donors, *FT* transformation of many genotypes is likely to be needed, and each sexual generation will need to be followed by phenotypic and/or genome marker evaluations—slowing breeding substantially. Gene editing would of course add to this complexity.

These techniques mostly depend on the florigen encoding gene *FLOWERING LOCUS T (FT)*, or associated floral regulatory genes (*e.g.*, *BpMADS4* or *CENTRORADIALIS/TERMINAL FLOWER 1*), that are overexpressed or suppressed (Flachowsky *et al.* 2009; Zhang *et al.* 2010; Flachowsky *et al.* 2011; Petri *et al.* 2018). To combine this technology with gene editing for transgene segregation in trees and clonal crops, an *FT* overexpression gene could be contained within the CRISPR/Cas9 T-DNA (Fig. 1). Another approach could include the grafting of CRISPR/Cas9 transgenic material with desired edits onto an *FT* overexpressing rootstock, as further discussed below. A more advanced form of these methods could be the generation of a Cas9 and *FTox* stable integrated line coupled with the viral delivery of desired gRNAs carried by systemic infection of an RNA virus such as TMV (Fig. 3). In this hypothetical case, a single transgenic line could serve as source material for the introduction of many different engineered traits. This would be highly desirable for species or genotypes with very low transformation rates—as is the case with many trees, other woody perennials, and clonally propagated crops.

One major challenge of employing strategies dependent on the grafting of transgenic materials containing editing transgenes is the graft transmissibility of FT protein, which has been shown in several instances when expressed *via* the commonly used 35S promoter *not* to be graft transmissible (Tränkner *et al.* 2010; Zhang *et al.* 2010; Zhang *et al.* 2010; Wenzel *et al.* 2013), with notable exceptions found in blueberry, *Jatropha curcas*, and citrus (Ye *et al.* 2014; Song *et al.* 2019; Soares *et al.* 2020). Expression from phloem-companion cell-specific promoters (*e.g.*, *SUCROSE SYMPORTER 2 (SUC2)*) rather than completely constitutive ones may increase the abundance of exportable FT protein to reach nearby grafted branches, as was recently shown in citrus (Soares *et al.* 2020). Presumably, this improvement is because although 35S overexpression of *FT* is able to induce early flowering through direct expression at the shoot apex, it is insufficient in the phloem-companion cells to be able to reach the thresholds required for flowering when transmitted through graft junctions. Further work is needed to establish the generality of such *FT* expression approaches for gene editing in trees and other woody perennial crops.

Recent Examples of Editing in Use in Trees and Clonally Propagated Crops, and Perspectives on Future Traits of Interest

A large number of engineered traits have recently been published in the literature or in conference abstracts in trees and clonally propagated crops. A summary of these recent demonstrations within the last 3 yr is available in Table 1 and includes members of 21 genera. At present, all reported demonstrations of CRISPR/Cas9 editing with the exception of two studies employing recombinase-mediated excision in apple and grape, and six instances of DNA-free Cas9-RNP delivery, had the editing transgenes stably integrated into the genome and there was no attempt to excise or segregate them away (Dalla Costa *et al.* 2020; Pompili *et al.* 2020). 19 of the 87 studies listed simply demonstrated the function of CRISPR/Cas9-mediated editing in a given species through mutation of a visual marker such as *PDS*. The types of traits targeted with the studied gRNAs included sexual reproduction, herbicide resistance, growth habit (dwarfism/semi-dwarfism), disease resistance, secondary xylem growth, lignin biosynthesis, onset of flowering, nodulation, concentration of toxic metabolites, starch or sugar accumulation, shelf life of fruits, biofortification, and metal transport. Of the 21 genera where editing has been validated, unsurprisingly, the most studied are in *Populus* (18), potato (15), *Citrus* (9), strawberry (9), and grape (6). Six of the 87 studies summarized in Table 1 used Cas9-RNPs for transgene-free gene editing, relying on protoplast regeneration and delivery via PEG or biolistic bombardment. These studies were in potato, grape, rubber tree, apple, and banana (Malnoy *et al.* 2016; Malnoy *et al.* 2016; Andersson *et al.* 2018; Fan *et al.* 2020; González *et al.* 2020; Wu *et al.* 2020). Two studies employed transient delivery of editing reagents by *Agrobacterium* without integration and only one succeeded to obtain edited, transgene-free, non-chimeric regenerants (Charrier *et al.* 2019; Bánfalvi *et al.* 2020). These studies were in potato and apple. No studies used virally delivered editing reagents in a tree or clonal crop. All the remaining studies used stable introduction of editing genes via *Agrobacterium*. The current method of choice for generation of transgene-free, edited lines is protoplast delivery of Cas9-RNPs, a technique which demands a large deal of technical expertise and is likely to be inaccessible for the vast majority of valuable tree and clonally propagated crop genotypes and species.

Regulatory Context for Gene-Edited Trees and Clonally Propagated Crops

US Regulation In the USA, the 1986 Coordinated Framework for the Regulation of Biotechnology ("Coordinated Framework") focused on regulating the process of

recombinant DNA ("rDNA") when used in plant and animal breeding (OSTP 1986). At first, the USDA-APHIS lightly regulated gene editing of crops under a "Am I Regulated" process (APHIS 81 FR 65414 2017). The focus was on whether the DNA had a plant pest gene and without such DNA sequences (*i.e.*, most of the crops submitted through this process), there was no legal hook for the USDA to regulate gene-edited crops at all (Martínez-Fortún *et al.* 2017).

In May 2020, the USDA-APHIS announced the Final Rule for its biotechnology regulations 7 CFR part 340, called the Sustainable, Ecological, Consistent, Uniform, Responsible, Efficient (SECURE) rule (APHIS, 2020). Thus, going forward, the USDA's Biotechnology Regulatory Services ("BRS") will implement this rule in assessing the environmental safety of biotech crops. The 2020 Rule defines genetic engineering broadly as "[t]echniques that use recombinant, synthesized, or amplified nucleic acids to modify or create a genome" (7 CFR § 340.3., 2021). Under USDA's new "Regulatory Status Review (RSR)" that is part of SECURE, developers can ask APHIS to evaluate novel plants and decide if those plants fall within the 2020 Rule's permit scope (but requires less data than the petition process it replaces, including an explicit statement that field trial data are not generally required). In a historically significant move, the USDA in this Rule focused on the product, not the process, used to make the organism.

The 2020 Rule also exempts certain new and old organisms from regulation. These include certain types of innovative plant breeding methods (of most note, genome editing when the change could have been obtained by conventional breeding), plant-trait-mechanisms of action that have already been approved (*i.e.*, MOAs, which are combinations of plant genera, gene functions, and traits) rather the individual gene insertions, and all "Am I Regulated" plants that were allowed under the past USDA process. In the future, the USDA could also exempt any plants whose modifications could have been achieved via conventional breeding. APHIS may grant an exemption itself or parties can request an exemption. APHIS will then provide public notice and opportunity for public comment if it agrees with the proposed exemption.

The rule also exempts minor DNA changes: (1) cellular repair of a targeted DNA break without an externally provided repair template, (2) a single deletion of any size, (3) natural DNA repair mechanisms, (4) targeted single base-pair substitutions (7 CFR § 340.1(b) (2), 2021), and (5) insertions from compatible plant relatives. Starting in August 2020, the USDA started letting developers request confirmation that their organism is exempt (normally within 120 d). Such confirmation letters will be posted on the APHIS website to "help them market their products domestically and overseas" (APHIS, 2020 at 29,799).

For regulated crops undergoing a RSR, if BRS finds no significant plant pest risk or other impacts after a review of the public comments under the National Environmental Policy Act (NEPA), the deregulation notice allows the developer to commercialize the biotech crop. The EPA also regulates those crops that resist herbicides (to approve herbicide uses and warnings) or pests covered under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). The FDA is expecting to continue to regulate genetically edited animals under its cumbersome veterinary drug approval process (Van Eenennaam *et al.* 2019) though in the final days of the Trump administration, the USDA attempted to transfer that regulatory role to them (Perdue 2021). After approximately 20 yr of regulatory requests, the FDA approved the AquaBounty® AquAdvantage® Salmon, highlighting the cumbersome nature of FDA regulation of animal biotechnology (Van Eenennaam and Muir 2011; FDA USF and DA 2019; Steinberg and Vittorio 2019). Many commentators are calling for a more reasonable approach than the FDA has taken with GE animals, particularly when there is no drug-related aspect (*e.g.*, a gene to prevent allergy that does not influence the structure and function of the animal or intended eater).

International Regulation The European Union's High Court of Justice in July 2018 ruled that crops and other organisms produced through genetic editing will be regulated as if they were a "GMO" under its long-standing "precautionary approach" to regulatory approval. This means that approval times will take several years, sometimes longer, for crops to be approved in the EU after being approved by nations planting the gene-edited crops (Callaway 2018).

The EU's "precautionary approach" is the law followed by the 171 nations that are parties to the Cartagena Protocol on Biosafety ("CPB"). These parties plan to meet again for the tenth time (COP-MOP 10) in early 2021 in Kunming, China. At MOP 9 in November 2018 in Egypt, the parties put gene editing under a "synthetic biology" descriptor. Since the USA, Canada, Australia, Argentina, and other grain exporting nations are not signatories to the CPB, we can expect parties to follow the lead of the EU, which is in many instances a key trading partner and source of foreign aid (*e.g.*, in parts of Africa, where the EU's influence on GMO policy is notably strong).

As part of the implementation of this law, nations that are parties to the Biosafety Protocol enact legislation, such as the European Traceability Directive, that imposes zero tolerance for the import of any GMO that lacks regulatory approval (The Cartagena Protocol on Biosafety to the Convention on Biological Diversity 2021). More nations are imposing regulatory approval requirements as the Biosafety Protocol is implemented. Thus, any biotech crop that could be exported, including gene-edited crops

or derived foods, may also require approval in many of these overseas markets. Similar problems exist for the potential use and export of GMO or gene-edited forestry crops (Strauss *et al.* 2009)

Canada regulates all "novel foods" and includes gene editing in that category. This encompasses those crops created using non-rDNA methods. For example, herbicide-resistant crops created using older methods such as chemical radiation, while newer methods such as gene editing, would be regulated. It is clear that those older forms of plant and animal "mutagenesis" breeding carry greater risks of "pleiotropic" changes (Wolt *et al.* 2016) that are often raised by activists opposed to gene editing (FOE F of the E (FOE) 2018) who see risks in off-target effects in genes (the adverse nature of which remain unlinked to health concerns). This is in spite of strong evidence that off-target mutagenesis from gene editing in crop plants (vs. animals) is negligible compared to other sources of natural and breeding-induced mutagenesis (Graham *et al.* 2020) Since many of these crops have similar ecological effects (*e.g.*, there are mutagenic, rDNA and genetically edited crops with herbicide resistance, all of which can outcross to wild relatives or cause problematic herbicide-resistant weeds to develop after widespread use), Canada's regime at least has a consistent approach to similar risks (Ellens *et al.* 2019).

In 2018, Argentina and 12 other nations, including other major grain exporting nations like Canada, Australia, Brazil, Paraguay, and the USA, issued a joint statement supporting agricultural applications of gene editing in agriculture ("precision biotechnology"), stating that governments should "avoid arbitrary and unjustifiable distinctions between end products (crop traits) derived from precision biotechnology and similar end products, obtained through other production methods." Signatories were Argentina, Australia, Brazil, Canada, Colombia, the Dominican Republic, Guatemala, Honduras, Jordan, Paraguay, the USA, Uruguay, Vietnam, and the Secretariat of the Economic Community of West African States (WTO 2018). We hope that common sense and good science will prevail over the arbitrary and capricious "precautionary approach" to regulating gene editing that may be applied to these products under the Cartagena Protocol on Biosafety (Nill *et al.* 2000).

In the USA, there are two routes for opponents of this technology to seek to stop the launch of a gene-edited crop, both of which relate to economic and environmental impacts related to the National Environmental Policy Act. First, injunctions to stop the launch of biotech crops have been granted against beets, eucalypt trees, and alfalfa for "interrelated economic effects" and forced environmental reviews after the USDA had conducted environmental assessments (but not a full environmental impact statement). Second, where there is an export-related economic interest involved, either an injunction under "anticipatory nuisance" (Grossman 2008) or post-marketing litigation seeking recovery for economic impacts to export-related interests (Ledbetter 2018). Depending on the trait modified and its connection to environmental and economic or market impacts, such routes may indeed be fruitful and delay their integration into the marketplace. To avoid such tactics, new laws that coordinate regulations across agencies in directing attention to comparative outcomes vs. use of recombinant methods, similar to what SECURE is hoping to achieve, might be needed in the USA. And to avoid trade confusion, similar new treaties are needed at the international level, whether inside or outside of the CPB.

Conclusions

Gene editing is growing rapidly in its application to trees and clonally propagated crops. The high efficiency that has been observed in annual crops clearly also applies to trees and clonal crops. The surge in publications and modified traits also suggests both a high scientific and a high commercial demand for its capabilities. However, the large majority of the publications to date have not had the gene editing machinery removed from the modified plants, confirming its difficulty in these crops. This is likely to be a result of their difficult breeding systems, delayed onset of reproduction, and general recalcitrance to transformation and regeneration of modified plants. Gene editing methods that do not insert transgenes, or seek to excise them after integration, though feasible and a subject of active research, have been rarely used and appear highly inefficient. We therefore believe that major innovations are needed both in methods for transient editing and excision, and in national and international regulation. Biological innovations should include the further development of widely effective viral systems for transient editing and efficient inducible recombinase systems for excision. Regulatory innovations should seek to focus on the products and their risk/benefit comparisons to conventional breeding methods, similar to that in the USDA SECURE system, thus not needlessly penalizing the use of recombinant DNA methods and the addition of inert (*e.g.*, non-coding) and/or well-studied and harmless DNA elements (*e.g.*, recombinase sites, T-DNA borders, common selectable markers) used when making new gene-edited varieties. Similar recommendations have been made many times with respect to transgenic crop regulation (*e.g.*, Strauss 2003; Bradford *et al.* 2005) Indeed, CRISPR-associated genes, even if left in trees or clonal crops used for food or feed, appear to have little potential for food safety or allergy problems (Nakajima *et al.* 2016), nor as discussed above as gene drives should they enter wild or feral populations.

Although the new USDA SECURE regulations provide several avenues by which trees or clonally propagated and gene-edited crops could be exempted from regulatory oversight or minimally regulated through the RSR process, serious regulatory and legal obstacles may remain given the uncertainty in how the

regulations actually become implemented in practice, and due to the limited harmonization among the USDA, EPA, and FDA in their gene editing rules (*e.g.*, in pest-resistant vs. other types of crops by the EPA vs. USDA). In addition, the NEPA process provides an avenue for opponents to greatly delay or block products, and the CBD is capable of blocking or greatly delaying all products intended for international markets. Given the great functional similarity of many gene-edited products with those from conventional breeding, gene editing is challenging regulatory systems to rethink their foundations, with increased emphasis on product over process, which scientists have recommended for GMO crops since the 1980s (National Academies of Sciences, Engineering, and Medicine 2016). With a decades-long record of GMO food and tree safety (Walter *et al.* 2010) and a world increasingly unable to produce its food and fiber sustainably, we hope that leaders take full advantage of this moment to craft more scientifically based systems, rather than cling to outdated method-based laws.

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