Tree breeding, a necessary complement to genetic engineering

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Abstract
The fields of tree breeding and genetic engineering can be perceived as being antagonistic towards each other—genetic engineers suggesting that tree breeding is too slow and expensive and tree breeders suggesting that genetic engineering is not practical and too expensive. We argue here that both fields have much to offer forestry and the success of each is intimately tied to the other. The major purposes of genetic engineering in forestry are described as well as the importance of evaluating tree engineering initiatives in the context of tree improvement and silviculture and integrating genetic engineering with tree breeding from start to finish. A generalized approach is developed that meets these requirements and demonstrates the interrelationships between the activities and phases of each program. In addition, a case study of the American chestnut (*Castanea dentata*) is provided to underscore the value of integrating genetic engineering and tree breeding programs to achieve a long-term conservation goal.

Keywords Forest genetics · Tree improvement · Biotechnology · Genetic modification · Reforestation · Restoration

Introduction

Tree breeding and biotechnology are tools or methods practiced within the field of forest genetics and tree improvement. Tree breeding is often viewed as the traditional approach to genetically improve trees, while biotechnology is seen as the modern and more technologically advanced approach. Tree improvement involves seed source testing and selection using provenance trials; parental testing and selection using progeny testing; and seed orchard establishment and maintenance for production of improved seeds and seedlings for reforestation (White 1987). The breeding or crossing aspect of tree improvement implies a multi-generational program producing a new generation of improved trees (selected parents for improved orchards) each breeding cycle. Ideally, the advanced generation of trees provides additional improvement or genetic gain in the target trait(s) when planted in the...
target environment(s) over the previous generation’s trees. Many variations exist on this theme and most utilize some form of biotechnology, including cloning selected trees in seed orchards for mass propagation of the improved trees. Cloning seed orchard trees is usually achieved by grafting selected genotypes onto non-selected rootstocks, while rooting cuttings is typically used for mass propagation. Clearly long-standing biotechnologies (i.e., grafting and rooted cuttings) are important components of most tree breeding programs.

Modern biotechnologies include tissue culture-based propagation, molecular-based genetic markers, gene cloning and sequencing, genome mapping and sequencing, genetic transformation/modification, and gene/genome editing. With respect to tree improvement, these biotechnologies can be grouped into three categories based on their use for conserving, assessing, or creating genetic variation, the raw material of genetic improvement: 1) various methods of propagation, including somatic embryogenesis, to conserve and leverage genetic variation; 2) genetic marker analyses to uniquely identify individuals, estimate relationships among genotypes, or improve the predictions of their performance; and 3) genetic engineering to add new or enhance existing genetic variation. Viewed through the lens of managing genetic variation there is wide consensus that modern biotechnologies are powerful tools in extending the impact of tree improvement (Campbell et al. 2003); however, when viewed through the lens of improving tree performance, the discussion usually centers on genetic engineering (GE) per se. For our current purposes, we consider GE or genetic modification (GM) to be any technique that uses recombinant, synthesized, or amplified nucleic acids to modify a genome and the resulting plants to be genetically modified organisms (GMO). To be clear, this will include plants that contain transgenes (genes from an unrelated species) as well as cisgenes (genes from a related species) and edited genes (e.g., in vivo genes modified with site specific nucleases, such as CRISPR/Cas9).

A common perception, especially among non-specialists is that with biotechnology, tree breeding can be at least partially by-passed with GE. And if not directly with GE, then by tissue culturing and mass producing only the very best, proven genotypes or using genetic markers to select the best parents without the need for long-term testing. These ideas, possibly taken out of context from the literature, are over-simplifications, but they fall into common usage and end up juxtaposing slow (expensive and long-term) tree breeding against fast biotechnology, undermining the ability of tree improvement to address pressing issues such as climate change and invasive pests. As presented in this paper, a more helpful and realistic perspective is developed for integrating biotechnology, specifically GE into tree breeding.

Of the modern biotechnologies, GE is a special case since its development and applications are regulated by national rules throughout the world. In the US, three agencies—Agricultural Plant Health and Inspection Service (APHIS), Food and Drug Administration (FDA), and Environmental Protection Agency (EPA)— have regulatory authority over GE. In agriculture, including forestry, APHIS through its Biotechnology Regulatory Service (BRS) serves a leading role focused on the potential for the GMO to present a plant pest risk (i.e., risk to plant health or to agriculture per se). Prior to 2020, APHIS regulated GMOs that were produced with plant pests (e.g., most commonly Agrobacterium-mediated transformation) based on the idea that introducing genes (i.e., the transfer-DNA from Agrobacterium’s Ti-plasmid) from known plant pests may result in an increased risk to plant health. Two types of increased risks of the GMO in the intended environment were typically considered—increasing susceptibility to a damaging insect or pathogen or increasing tolerance to standard weed control measures. Specifically, based on recent determinations of non-regulated status (e.g., Pioneer DP202216 Maize and Monsanto MON 88,702
Cotton, information available on BRS’s web site), APHIS appeared to be interested in the following aspects of plant pest risk:

- Potential of the GMO as a risk to plant health or a change in its metabolism due to the transformation process or expression of new genetic material
- Disease or insect incidence or damage to the GMO in the intended growing areas and its potential impact on APHIS’s pest control programs
- Impact to beneficial, non-target organisms by exposure to or consumption of the GMO
- Weediness of the GMO and its potential impact on weed control practices
- Impact of the GMO on weediness of other related plants that it may interbreed with (gene flow, vertical gene transfer)
- Impact of the GMO on common agricultural practices (e.g., pesticides, fertilizers, herbicides, tillage, etc.)
- Potential for horizontal gene transfer (from the GMO to an unrelated organism) and impact of the transfer on the other organisms’ potential to cause damage (e.g., as a pest, pathogen, parasite, etc.)

Enacted by APHIS in 2020, the SECURE rule provides an updated and more efficient method for determining whether a GMO will be regulated. The efficiencies come in a few ways including the potential for exempting certain GMOs, the consideration of the engineered plant only (not individual transgenic events) with respect to its potential to increase plant pest risk, and an abbreviated process for determining whether an engineered plant poses an increased plant pest risk. The first efficiency allows for a developer to apply for an exemption from regulation for certain categories of engineered plants, including a plant with a single modification that could have been produced with conventional breeding or a plant with a plant-trait-mechanism of action (MOA) that APHIS has already determined to be unlikely to pose an increased plant pest risk. The second and third efficiencies allows for the developer to request a regulatory status review (RSR) to determine if the GMO will be regulated. The RSR evaluates the engineered plant—specifically the plant’s biology, the new trait, and the trait’s MOA—and not individual transgenic events as was the case under the previous rule.

The EPA and FDA regulate GE to ensure human and environmental safety and food and feed safety, respectively. Specifically, the EPA regulates pesticides, including those termed plant incorporated protectants (PIP) that are engineered into plants, through a registration process. To gain registration (i.e., a license to market), the GMO producer must show that the PIP is effective and poses no elevated risk to human or environmental health when used according to label. The label is developed to manage risk by defining who can use the PIP, as well as where, when, how much, and how often. In addition, EPA labeling mandates the implementation of an insect resistance management (IRM) plan to protect the efficacy of the PIP (i.e., to slow the development of resistance to the PIP). The FDA regulates most human food and animal feed, including those produced by or containing GMOs. If nuts or fruits of GE forest trees are to be consumed as food or feed, the FDA must be consulted to ensure that these materials meet the same safety standards as comparable non-GE products. The same applies with respect to the EPA, for GE forest trees that contain a PIP for insect, disease, or herbicide resistance.

In addition to the technological and regulatory aspects of GE in forestry, there are social and ethical dimensions (Neumann et al. 2007; Garcia-Yi et al. 2014; NASEM 2019; Petit et al. 2021). What is the impact of the new biotechnology on the local community overall as well as various subgroups and even individuals? Will the community accept
the application of the new biotechnology in their local area? What impact may forest certification systems have on the application of new biotechnologies (Strauss et al. 2015)? Although one major certification system has begun a "learning process" to examine possible future allowances for GMOs (https://fsc.org/en/sustainable-intensification/fsc-genetic-engineering-learning-process), at present, no certification system allows GE trees of any kind, even with complete gene confinement. This will likely impact the use of GE on non-certified lands due to the potential, eventual spread of reproductive material (vegetative, pollen, or seeds). For GE to be successful in forestry, all parties will need to be informed as well as given the opportunity to voice their concerns (NASEM 2019). This happens to a degree, at a very broad level within the regulatory process, but the effort will need to be expanded as on-the-groundwork begins in terms of tree planting and managing the restoration effort. Within the societal sphere of their restoration model, Jacobs et al. (2013) include regulatory and policy issues but also point to cultural and economic values and collaborative networks converging towards a common understanding of the restoration effort, including public acceptance and ownership of the technology and the restoration goals and management techniques (Fig. 1). This holistic restoration model, that merges the technological (including breeding and genetic engineering), ecological, and societal spheres, provides a framework of how a successful restoration program might look as it begins to meet its goals and objectives.

It is within this context of developing and utilizing biotechnology, or more specifically genetic engineering, in forestry that we consider tree breeding and how it complements and is required by GE in both its development and application. We will consider in turn, the purposes of GE in tree improvement, a generalized approach for developing and deploying GE traits in forestry, and a case study of GE American chestnut in ecosystem restoration.

### Purposes of GE in tree improvement

Genetic engineering has provided value and found wide-spread use in breeding major agricultural crops in the USA by adding or enhancing specific traits (Fernandez-Cornejo et al. 2014). In various cases, these traits include herbicide tolerance (add), insect or disease resistance (add/enhance), drought or salinity tolerance (enhance), quality traits (add/enhance), and yields (enhance); however, the genetic complexity of the latter, more complex traits have largely kept commercial successes out of reach (Chan et al. 2020). Some of these traits increase the efficiency of farming and may decrease or increase the amount of chemicals used and others increase the value or quantity of the products produced. In each case, the breeder must determine the objective of the GE intervention and how it will be integrated into the available gene pools, breeding lines, and ultimately commercial varieties. Genetic engineering is clearly a breeding tool, in some respects like more familiar tools such as mutation screening, inbreeding, wide-crossing, introgression, and marker-assisted selection, but also different in that it is a direct and specific intervention of the crop species’ DNA. All these ideas and concepts are relevant to forest trees (Harfouche et al. 2011; Chang et al. 2018), but here we will focus on the two primary purposes of GE that are most relevant to contemporary forestry—species rescue and restoration and improved plantation performance (e.g., growth rate, product yield and quality, management efficiency). These broad applications are primarily distinguished by the silviculture and ecology of the forest systems, including regeneration, stand management, and regeneration harvest. In species rescue and restoration, we are typically considering natural regeneration,
uneven-aged stand management, and partial cutting for regeneration. While for plantation performance, the system is typically artificial regeneration, even-aged management, and clearcutting for regeneration. Afforestation can be considered a special case of either purpose in which the forest is initiated by artificial regeneration of a site without trees. In all cases, the necessary trait is added, subtracted, or modified in some way to achieve the goal of the intervention for modifying tree performance.

Several forest tree species have been or are being decimated by invasive species, typically an insect pest or fungal pathogen, and often these invasive species are introduced
(non-native) and thus have not co-evolved with their hosts. Native species can have limited to no resistance to introduced pests and pathogens, since their separation during speciation has prevented them from co-evolving effective resistance mechanisms (Thompson and Burdon 1992). Typically, the pest or pathogen causes minimal and manageable damage on native species in native environments while causing extensive damage and possibly widespread death of related species in the new environment. We have seen this in several forest trees in North America in the last century. Well known examples include American chestnut (chestnut blight), American elm (Dutch elm disease), and the white pines (white pine blister rust). Lesser known and more recent examples include the hemlocks (hemlock woolly adelgid) and ashes (emerald ash borer) of eastern North America, butternut (butternut canker), American beech (beech bark disease), red bay and sassafras (laurel wilt), and Florida torreya (Fusarium canker disease). Given time, selective breeding or even natural selection may provide the native tree species with enough resistance to survive, reproduce and co-evolve with the invasive pest or pathogen. In the meantime, the landscapes, ecosystems, and society continue developing with the species being functionally or even completely extirpated from the environment.

Within this context, the question becomes, can GE provide these threatened trees with resistance to introduced pests and pathogens in a timely manner, such that the species can be saved and restored to landscapes and ecosystems and what would that look like? One example (discussed later) is the American chestnut (Castanea dentata), where GE has provided a new possibility for species restoration (Jacobs et al. 2013; Newhouse et al. 2014). The combination of GE and species restoration is unique, in that in all previous cases of GE in agriculture, horticulture and forestry the engineered plant or crop is not expected to establish itself (regenerate on its own) in the environment. In fact, GE crops are specifically designed to prevent persistence and reproduction in the environment. This is different for species restoration, where the engineered trees’ purpose is to persist in the environment, establish a self-sustaining population and in some cases spread the GE event to progeny of extant trees in the environment. For forest restoration, self-sustaining populations is an essential purpose of GE in trees, and the discussion will force society to decide whether we should let a species be reduced to the margin or even disappear when GE provides a solution to saving and restoring the species?

To date the primary objective of GE for forestry has been in adding a specific trait or enhancing trait variation (Chang et al. 2018) for improved performance in plantations. The context in these cases is similar to agriculture where a specific crop is intended to be planted, managed, and harvested with little opportunity for natural regeneration. The possibility for natural regeneration is eliminated in the GE development phase by physically removing flowers and seeds prior to pollination/fertilization and seed maturation, or by using single genotypes that are either self-incompatible, the same sex (dioecious species) (Lorentz and Minogue 2015) or engineered to be sterile in combination with the target trait (Brunner et al. 2007; Zhang et al. 2012; Klocko et al. 2016a). The engineered approach, especially sterility, is likely more robust as gene flow into compatible related species that share the immediate area can be prevented. A variation on the single genotype approach is where the engineered trees are a non-native species, and they are completely unrelated (non-sexually compatible) to any species in their new environment (e.g., Lorentz and Minogue 2015). The use of GE for trait addition or enhancement is obviously most applicable for plantation-grown species managed in discrete rotations, where each rotation is initiated by planting and terminated with a clear-cut harvest followed by site preparation and planting the next rotation. In addition to traditional forest tree plantations for wood products (e.g., pines, spruces, Douglas fir, eucalyptus), many other species and systems can
be considered from agroforestry to short-rotation biomass, conventional rotation softwood timber, and long rotations of high-value hardwoods.

Classes of traits usually considered for addition or enhancement include herbicide tolerance, insect or disease resistance (biotic stress), drought, flood and salinity tolerance (abiotic stress), wood properties, and growth rate. Addition of herbicide tolerance is a desirable trait in hardwood plantation culture as effective weed control is critical for productivity and difficult/costly to achieve (Meilan et al. 2002). In addition, the technology is readily available for some of the more popular herbicides (Werck-Reichhart et al. 2000; Zhang et al. 2019). Addition or enhancement of biotic stress resistance or tolerance is a necessary trait in situations where the insect or disease completely limits the productivity of the plantation species. In some cases, breeding has been effective (Schmidt 2003; McKeand et al. 2003; Sniezko and Koch 2017; Pike et al. 2021), but in other cases natural variation in resistance is apparently not available and would need to be added through GE. Even though the target trait can be relatively well-defined, the variety of mechanisms producing the trait variation are complex and thus these traits can be difficult to modify with GE. Continued work on mechanisms of resistance and the genes that underpin them will improve this situation.

The enhancement of tolerance to abiotic stress is an important feature of tree performance in stands planted for wood/fiber production (Harfouche et al. 2014). This is especially the case for plantation management under a changing climate, where enhanced drought, flood or salinity tolerance may be needed to maintain adequate survival and growth rates. Modification of wood properties is a promising target of GE (Halpin and Boerjan 2003; Mansfield et al. 2012; Ralph et al. 2019). Changing the ratio of the lignin sub-units is important for pulping efficiency as is changing the ratio of cellulose to lignin (Chanoca et al. 2019). These traits are readily modified with GE given the current in-depth understanding of the metabolic pathways involved in lignin and cellulose biosynthesis (Boerjan et al. 2003; Chiang 2006). Finally, growth-related traits are often considered, and these efforts may be directed towards increased efficiency in water-, nutrient- or light-usage to provide improved wood and biomass yields and overall carbon sequestration. Growth and yield traits tend to be genetically complex and thus are difficult targets for GE, although improvements would provide economic value of the tree crop (wood yield) as well as improved ecosystems services through increased capacity for sequestering carbon and increased growth efficiency under varying environmental conditions.

The potential applications of GE in forest trees ranges from species rescue and restoration to production efficiency and wood yield and quality gains in species used in plantation forestry. As discussed, specific applications within these two categories have different perspectives on the traits to be engineered, the genotypes to be deployed, and the management system planned. Implementation of GE in the breeding program, invokes questions such as, what trait variation provided by GE is needed to achieve the breeding objectives; in what genotypes will the GE trait be deployed for the field; and will the GE trait be transmitted to the next generation through natural regeneration or contained within the current set of planted genotypes? In the next section, we will discuss a generalized approach for implementing GE in tree improvement. This approach requires determination of the biotechnologies necessary to produce GE trees, the stages of screening for trait efficacy and off-target effects, and the steps needed for increasing the selected GE events in trees appropriate for deployment within the target environment under the planned management system. Regulatory and societal considerations are in play throughout the GE development process (Viswanath et al. 2012; Petit
et al. 2021), and these will be highlighted at relevant points in the section discussing American chestnut as a case study.

**Generalized approach for GE in tree improvement**

(A) Develop transformation system for species of interest

A GE initiative typically begins with an evaluation of the silvicultural problem (or opportunity) and options for solving it, including without and with GE. The Forest Health Initiative (FHI) produced a roadmap (Fig. 2) for such an analysis of forest health problems (Nelson et al. 2014). In addition, an integrated impact assessment framework has been developed to evaluate biotechnological options for addressing threats to forest health (NASEM 2019, chapter 5). Both approaches could be adapted for issues in silviculture and tree improvement beyond forest health. Assuming GE is considered a favored alternative, the generalized approach developed here (Fig. 3) starts with establishing a transformation system (e.g., technology for incorporating the gene of interest into the tree) for the species of interest (Fig. 3, orange cycle). This could be done through collaboration or be brought inhouse, but with either option the transformation work must be closely integrated with the breeding program (Fig. 3, blue cycle).

![The Forest Health Roadmap](image)

**Fig. 2** The forest health roadmap as developed by the Forest Health Initiative. The three phases move from a characterization of the threat to analysis of various options, including from technological, social, and ecological aspects to exploration of various biotechnology-based solutions if required. The responsible use principles, as developed by The Institute of Forest Biotechnology (Costanza and McCord 2011), underpin the biotechnology considerations.
Most notably, this would include the choice of genotypes used for transformation and an understanding of the protocols to be used for screening the candidate GE events. For the program to be successful, the transformation system must have established protocols from tissue culture through to whole plant regeneration (Kong et al. 2014). High-quality tissue culture leads to increased efficiencies at getting cells transformed and transformed plants ready for screening.

(B) Screen germplasm for transformability and secondarily early flowering

Optimally, the available germplasm is initially screened for transformability and early flowering. Transformability includes being put into tissue culture, transformed, and regenerated into plantlets and plants. The transformed lines are a means of transmitting the GE event into the appropriate untransformed genotypes through controlled pollination, and this crossing, especially in trees, on genotypes that have the capacity to flower early. Additionally, for dioecious species it is important to consider the sex of the genotypes being screened and whether transformed genotypes of both sexes will be needed. If using somatic embryogenesis as the tissue culture method, immature embryos from a diverse set of trees (including those known for early flowering) within the breeding program are evaluated for transformability, with the best transforming lines being regenerated into plants and tested for early flowering under flower inducing conditions. A few unrelated genotypes that pass these two screens provide an optimal set of “workhorse” lines for the GE initiative (Fig. 3, orange cycle). In addition, GE could be used at this stage to produce early flowering (Klocko et al. 2016b) workhorse lines. With this addition, the early flowering trait (i.e., transgene) would need to be
selected against when selecting lines for deployment through seed or clonal propagation (see stage F). This approach, termed rapid cycle breeding, is also proposed as a method to speed tree breeding in general (Callahan et al. 2016; Flachowsky et al. 2011), especially when paired with marker-assisted (Ribaut and Hoisington 1998) or genomic selection (Isik 2014). Furthermore, the use of early flowering genes with viral vectors may simplify this approach since viral vectors are often not transmitted to the next generation (Yamagishi et al. 2014).

1 (C) Test candidate genes for expression and trait performance in best transforming lines

Once the workhorse lines have been selected (Fig. 3, orange cycle), candidate gene testing can begin. A discussion on evaluating specific strategies for engineering the trait and selecting candidate genes is beyond the scope of this paper. Instead, our purpose is to generalize the approach and discuss how the GE initiative interfaces with components of the tree breeding program, namely testing, selection, and crossing (Fig. 3, blue cycle). Candidate gene testing includes evaluating the gene construct (the gene and its promoter) in a specific set of GE events, in a small number of workhorse lines. A GE event is one candidate gene construct in one transformation occurrence (event) in one workhorse line. Given that the genome location of the transformation event can strongly influence the expression of the gene, the expression of the candidate gene is best tested in a large sample of GE events. A sample of the highest expressing GE events are then tested for candidate gene copy number and preferably only those with one insertion location are selected for further work. High expression and single insertion location are both important at this stage and serve as a first good filter for reducing the GE events for further testing.

The next stage is to evaluate the trait in the selected GE events. To do this, the selected events will need to be regenerated into plants as the trait expression at the whole-plant level is generally the desired outcome of the transformation. Those plants representing GE events that have adequate trait expression can be further evaluated for early flowering. Depending on the purpose of the GE end-product, selecting good trait expression and early flowering will facilitate the later stages of the GE initiative. Conceptually this is relatively straight-forward but having an accurate early-testing protocol for trait expression is necessary, as well as the ability to test for early flowering. The latter can be done under conditions optimized for flowering, especially if that environment can be used in later stages of the initiative. In addition, the region of the genome surrounding the GE event should be sequenced to determine the genome context and the integrity of the candidate gene insertion. At this point, we have GE event(s) that need further evaluation and test trees can be produced in the workhorse lines with continued propagation. Concomitantly, test trees should be produced through seed, by crossing the GE trees with genotypes selected from the breeding program (Fig. 3, blue cycle). These first crosses are important to both evaluate the stable transmission of the GE event to progeny and to produce progeny trees for further testing of gene expression and trait performance.

(D) Test GE events for expression and trait performance in representative genotypes and environments

The testing component (Fig. 3, blue cycle) may be viewed analogously to pre-clinical and clinical trials in drug or vaccine development. In a GE initiative for trees, the earliest testing is analogous to pre-clinical trials, including the testing described above, and can be considered lab (or pre-tree) trials. Tree- and forest-testing comprise the phases analogous to the clinical trial’s phases 1 and 2 (tree testing) and phases 3–4 (forest testing). Phase 1 looks at how the gene works in workhorse genotypes, includ-
ing studying potential adverse effects on the tree. Phase 2 determines how the trait is expressed in the workhorse lines and whether the desired phenotype is obtained. As performance information is gained in these greenhouse- and confined field-based experiments (phases 1–2), the list of candidate GE events can be reduced so larger, longer-term field experiments (phase 3) can be conducted. These experiments are much larger studies looking at how the gene and trait perform in different genotypes and environments. Trees produced by the first crosses mentioned above can be utilized in phase 3, assuming crosses are made with a variety of parents, ensuring that the gene and trait can be evaluated in diverse genotypes. In addition, the parents used should be pre-selected to be adapted for the intended test environments. The initial crosses are ideal materials for evaluating the events in phase 3 experiments, since with each cross about half the progeny will be GE-positive and half GE-negative. The GE-negative trees serve as the placebos in the drug/vaccine trial analogy.

Continuing the analogy, phases 1–3 are conducted during APHIS’ regulatory review. Phase 4 trials commence once regulatory approval is reached and these trials include reporting on the gene and trait performance in the intended application (background genotypes and target environments). Phase 4 trials can be used to validate that the gene and trait are safe to the trees and the environment (including humans and wildlife) and that the gene is effective at providing the desired trait (phenotype), and to determine the optimal use (trait-environment combination) of the gene/trait. It is important for phases 3 and 4 to be conducted in the environment as intended for long-term application (e.g., species restoration, plantation forestry). In terms of increasing available planting stock for phases 3 and 4 testing, the basic idea is to establish the GE event in genotypes that are representative of the planting stock being planned for deployment. In the case of species rescue or restoration, this could be T1 or T2 generation (first and second generation after the GE event) seedlings prior to the intercross generations as described by Westbrook et al (2020). For deployment plans in forest plantations (conventional or short rotation), the T1 and T2 generation crossing should emphasize the use of high-performing (elite) parents as testers where the planting stock is either seedlings in the case of full-sib (FS) family seedling deployment or vegetatively propagated stock where clonal deployment is planned. Alternatively, it may be best to defer phase 4 testing until the final products for deployment are available and are increased to a level needed for these trials.

(E) Incorporate selected GE events into the breeding program

The planned deployment strategy drives the breeding program during phases 3 and 4 testing (Fig. 3, blue cycle), and three basic possibilities exist, depending on the purpose of the GE initiative. First is crossing with diverse and adapted germplasm for multiple generations to produce self-sustaining populations for the species rescue and restoration effort. Second is to cross with elite germplasm adapted to the target environments and then select GE-positive, high performing clones for continued clonal propagation and deployment. Third is like the second, except with an addition of an option where the FS families are deployed as seedlings. The main goal of the breeding program is to minimize the GE founder genome among the parents in successive generations, including the portions linked to the GE event. This is similar to classical backcross breeding where a single gene is being transferred (or introgressed) from a donor genotype (the workhorse line) into the recipient genotypes (elite parents). Marker-assisted selection can be used to optimize the recovery of recurrent genome at each generation (Ribaut and Hoisington 1998), reducing the number of generations needed to recover and identify elite parents with the GE event. In the first case, where an open-pollinated (OP)
seed orchard is needed to produce planting stock for deployment, three to five backcross generations is recommended, depending on the number of founders and parents used in each generation (Westbrook et al. 2020), followed by an intercross generation. The intercross generation will produce trees that are homozygous (or true breeding) for the GE event. In the second option, cloned genotypes within each backcross generation can be evaluated for desired performance. An intercross is not needed since the selected clones will be directly deployed. In the third option, intercrosses can be made at each backcross generation to obtain selections that are homozygous for the GE event. These trees can then be evaluated as parents for elite cross-pollinated (CP) families. If the workhorse genotypes came from elite CP families only one or two backcross generations may be needed to recover elite performance.

(F) Produce GE event-positive planting stock for deployment

Generations can be advanced within the phase 3 testing program (Fig. 3, blue cycle) in a similar way as conventional breeding programs advance generations of cycles of testing, selection and crossing. Thus, phase 3 testing serves the dual role of evaluating the GE event in a variety of genotypes and environments as well as providing advanced generation selections. As the final selections are made, seed orchards or stool beds for the deployment population (Fig. 3, green component) are established with the planting stock going into phase 4 trials. Optimally this will be timed with achieving non-regulated status for the selected GE events or traits. Special considerations for the seed orchard design may be needed depending on the agreed post-regulatory reporting commitments. For the cases of plantation forests this would most likely be an agreement to impose practices that limit gene flow of the GE event into the wild type populations. For the case of species rescue or restoration, it could be limiting seed production until phase 4 results confirm expectations derived from the phase 3 trials. In both cases, consideration of managing the GE event in planting stock production revolves around the sex of the GE event genotypes. For seed production (including immature embryos for SE) in monecious species this requires managing the GE event genotypes as females for seed, males for pollen, or for both seed and pollen production. Another consideration is the source and diversity of the organelle genomes in the planting stock. If the seed orchards are formed with parents after only one or two generations of backcrossing, organelle diversity could be low as these genomes do not recombine. Thus, the number of parents used as both females and males defines the level of diversity in the planting stock. Beyond these considerations the process is much the same as conventional programs where the orchards or stool beds are scaled (number of ramets or clonal copies of each selected ortet or genotype) to the level of expected demand.

(G) Alternative approach utilizing pre-selected elite clones for transformation

The alternative described here is to focus the initial transformations (Fig. 3, TG event production) on elite-performing clones and select and clonally increase the best TG events for phases 3 and 4 testing, and once-proven, commercial production (Fig. 3, Increasing for phase 3–4 trials and Increasing for deployment and Outputs). This strategy has been discussed in various forms and is being developed and deployed in some cases (Harfouche et al. 2011; Chang et al. 2018). The work leading up to the selection of the elite clones falls within the context of tree breeding even if only clonal testing was completed (i.e., no crossing and among- and within-family evaluations). Furthermore, the alternative falls within the generalized approach, that is to say, integrated with the tree breeding pipeline, with the exception of clonal testing the TG events instead of testing through sexual progeny. The clonal selection of TG events can lead
to selected GE clones for direct deployment in clonal plantations or through use in seed orchards if cleared for release by the regulatory agencies. The persistent issues with this alternative are difficulty in transforming and propagating some or even most of the elite clones (Chang et al. 2018) and potentially having too little genetic variation in the population to be deployed. The later issue is particularly important for most forestry applications due to the long rotation times and heterogenous environments. Clearly, high genetic diversity is desired for species rescue and forest restoration (Westbrook et al. 2020).

**Summary of generalized approach**

The generalized approach for utilizing GE in forest tree improvement (Fig. 3) highlights the importance of tree breeding per se and the need for integration and coordination from project planning through implementation. A GE initiative is not a short-cut to tree improvement, but an enhancement, providing new possibilities to address age-old and emerging problems. The initial question—can GE solve the specific forest conservation or management problem at hand more effectively and efficiently than existing methods—needs to be addressed. If the answer is yes, then several follow-on questions are needed to be asked and answered before the specifics of a plan can emerge. In all cases, the planning must be integrated with the ongoing tree breeding program to ensure project success. Technical, regulatory, and societal issues need to be addressed in advance and continually re-evaluated during project implementation (Jacobs et al. 2013). In the next section, we present a case study of American chestnut, considering how breeding and GE developed independently to solve the same problem only later to become integrated to improve the chances of success in terms of species restoration.

**Case study, GE American chestnut for restoration**

American chestnut, once known as the king of the Appalachian forest, has been functionally extirpated from the forested ecosystems of its native range (Newhouse 1990; Dalgleish et al. 2016) by a non-native fungal pathogen (*Cryphonectria parasitica*) causing chestnut blight (Anagnostakis 1987). Breeding efforts have been ongoing for essentially the last century, some looking to develop blight resistance within the native gene pool (Griffin 2000) and some looking to obtain resistance from related species through hybridization (Burnham et al. 1986; Diskin et al. 2006; Anagnostakis 2012). The former approach depends on finding and breeding large surviving trees, inferring that these trees have a rare combination of genes that provides an adequate defense against the pathogen. The latter approach coming in two forms, one suggesting that a hybrid chestnut is necessary to achieve enough resistance and is likely the best than can be done, and the other suggesting that by back-crossing the hybrid to American chestnuts and selecting for resistance in successive generations a tree close to the American chestnut can be obtained with adequate resistance to restores the species. Although some level of success has been achieved with these methods, none have provided the trees necessary for a concerted effort designed to restore the species in the native landscapes. This is likely due to a few factors including lack of confidence in the resistance and uncertainty over the silvicultural methods needed to reintroduce American chestnut using artificial regeneration. Confidence in resistance has a couple perspectives as well—is the resistance strong enough to protect the tree for a full biologic
rotation (more than 100 years), allowing for decades of flowering and seed production? And is the resistance durable enough in terms of maintaining an adequate level of protection against a pathogen that is evolving? These questions obviously can’t be answered, but they need to be asked as part of an adaptive management strategy for restoration.

While forest tree breeding is well established, GE in forest trees (reviewed by Chang et al. 2018), including American chestnut (Polin et al. 2006; Andrade et al. 2009) is relatively recent. The technologies required were adapted from model and crop plant biotechnology primarily by labs at the University of Georgia (UGA) and the State University of New York—Environmental Science and Forestry (ESF) (Carraway and Merkle 1997; Andrade and Merkle 2005; Polin et al. 2006; Andrade et al. 2009; Newhouse et al. 2014). The ESF effort has culminated in the development of a GE American chestnut, named Darling 58, that exhibits high blight resistance in greenhouse and short-term field tests under artificial and natural inoculation (Newhouse et al. 2014; Newhouse 2020). In the case of Darling 58, a petition for non-regulatory status is under consideration at APHIS that would clear the way for its use in the environment. This would be the first case in which GE has been used for the restoration of a species to its natural environment. Darling 58 contains the transgene OxO from wheat (Dratewka-Kos et al., 1989; Liang et al. 2001) that provides protection to American chestnut by neutralizing the pathogen’s oxalic acid that it uses to injure and defeat the trees’ defensive reactions leading to canker formation, eventual stem girdling and dieback. The transgene is expressed in a single wild-type genotype of American chestnut originating from New York state. Thus, Darling 58 was developed without the use of tree breeding. Essentially the mechanism of the pathogen’s success at causing cankers was discovered, a gene that could counter that mechanism was identified, and introduced into an available American chestnut genotype that was subsequently propagated in tissue culture for testing at the whole plant level. At this stage, the gene, its introduction into a workhorse genotype, and this combination’s efficacy at preventing disease in short-term tests has been established, without tree breeding, but from this point forward, successful application in species restoration will require tree breeding as described by Steiner et al. (2017) and Westbrook et al. (2020).

In general, the breeding steps include crossing the GE genotype to several wild-type American chestnut trees over a few generations, and each generation selecting Darling 58-positive plants to serve as parents for the next generation. Carefully selecting wild type trees from local or regional populations provides a population that is diverse and adapted to the regional environment. Many new parents are utilized each generation to reduce the founder effect of the original GE parent and achieve adequate diversity for evolution to act on (Westbrook et al. 2020). In the case of the American chestnut, the collaboration between the GE team at ESF and The American Chestnut Foundation (TACF) and its state chapters has provided the structure for carrying out such a post-GE initiative breeding effort. Concurrently, TACF continues to utilize a backcross breeding strategy to introgress resistance from Chinese chestnut (C. mollissima) into American chestnut. Trees from the backcross generations of breeding can also be used as parents in crossing with the GE tree to develop the diverse and adapted populations for restoration. Through the technical approach of combining breeding, biotechnology, and biocontrol, the increasing societal acceptance through the public support of environmental and conservation groups (e.g., Sierra Club; Melton 2021), and ESF’s commitment to the regulatory process, the American chestnut is on the verge of becoming an inspiring success story in species rescue and restoration. The three spheres (technology, ecology, and society) of the Jacobs, Dalgleish, Nelson (JDN model, Fig. 1)
restoration model (Jacobs et al. 2013) are largely converging and predicing increased probabilities of success in restoration of the American chestnut.

**Conclusion**

Genetic engineering offers unique possibilities to forestry when considered as an integrated component of tree improvement and the planned silvicultural system. In this way, GE may be considered when traditional alternatives are not sufficient to meet management objectives. Categories of these objectives include tree species rescue and forest restoration, and improving species performance (e.g., productivity, management efficiency, economics) in plantation forests. However, it is important to emphasize that GE is not a short-cut to tree improvement. Long-term planning and commitment are needed for success starting with evaluating alternatives and engaging stakeholders, moving to lab- and greenhouse-based R&D, short- and long-term field testing, regulatory review, increasing the proven de-regulated GE-trees, and planting and managing the trees in the environment. As an example, American chestnut may prove to be the first forest tree species rescued and restored utilizing GE technology. Although, not explicitly integrated with tree breeding from the start, the GE and breeding programs are now working closely to ensure the GE-events are being bred to combine disease resistance, genetic diversity, and regional adaptability. As the management and conservation challenges continue to arise across forestry, tree improvement can be well-positioned to respond by embracing and anticipating new technologies and developing novel approaches to improve the likelihood and timeliness of breeding trees that meet and exceed management objectives.

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**Declarations**

**Conflict of interest** CDN declares no conflicts of interest.

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