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INVITED REVIEW

# Genetic engineering of trees: progress and new horizons

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## Abstract

Genetic engineering of trees to improve productivity, wood quality, and resistance to biotic and abiotic stresses has been the primary goal of the forest biotechnology community for decades. We review the extensive progress in these areas and their current status with respect to commercial applications. Examples include novel methods for lignin modification, solutions for long-standing problems related to pathogen resistance, modifications to flowering onset and fertility, and drought and freeze tolerance. There have been numerous successful greenhouse and field demonstrations of genetically engineered trees, but commercial application has been severely limited by social and technical considerations. Key social factors are costly and uncertain regulatory hurdles and sweeping market barriers in the form of forest certification systems that disallow genetically modified trees. These factors limit and, in many cases, preclude field research and commercial adoption. Another challenge is the high cost and uncertainty in transformation efficiency that is needed to apply genetic engineering and gene editing methods to most species and genotypes of commercial importance. Recent advances in developmental gene-based transformation systems and gene editing, if combined with regulatory and certification system reform, could provide the foundation for genetic engineering to become a significant tool for coping with the increasing environmental and biological stresses on planted and wild forests.

**Keywords** Trees · Forest biotechnology · Genome editing

## Introduction

It has been more than 30 yr since the first report of a transgenic tree (Fillatti *et al.* 1987). In the intervening years, hundreds of transgenic constructs have been transformed into forest trees, most frequently varieties of poplar (*Populus*) species and hybrids, but including several other genera such as *Acacia*, *Betula*, *Eucalyptus*, *Picea*, and *Pinus* (see reviews by Confalonieri *et al.* 2003, Merkle and Narin 2005, Nehra *et al.* 2005). Despite genetic engineering (GE) technology being a boon to research on tree biology, the commercial use of transformed trees remains very limited. In 2002, two lines of *Bacillus thuringiensis* (*Bt*) toxin-producing poplar were authorized for commercial planting in China (James 2015), and in 2015 FuturaGene obtained permission for the commercial release in Brazil of a single line of transgenic *Eucalyptus* with improved growth (Anonymous 2015). This is in sharp contrast to the rate of adoption of GE technology in agricultural crops. Fertile GE soybean and maize were first reported at approximately the same time as poplar (Hinchee *et al.* 1988; Gordon-Kamm *et al.* 1990), and consequently, transgenic lines of these and several other row crops were approved for commercial release in the USA in the mid-1990s. Since then, many other approvals carrying single or stacked input and output traits have been approved.

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Despite many challenges, tree biotechnology has made significant progress in recent years. Reference genome sequences have become available for commercially valuable timber trees such as *Populus trichocarpa* (Tuskan *et al.* 2006), *Eucalyptus grandis* (Myburg *et al.* 2014), spruce (*Picea glauca* and *Picea abies*) (Birol *et al.* 2013; Nystedt *et al.* 2013), *Pinus taeda* (loblolly pine) (Neale *et al.* 2014), *Pseudotsuga menziesii* (Douglas-fir) (Neale *et al.* 2017), and *Fraxinus excelsior* (European ash) (Sollars *et al.* 2017). Genomes of several commercially important fruit or specialty trees such as *Malus domestica* (apple, cv. Golden Delicious) (Velasco *et al.* 2010), *Prunus persica* (peach, cv. Lovell) (The International Peach Genome Initiative 2013), *Citrus sinensis* (sweet orange) (Xu *et al.* 2013), *Theobroma cacao* (cocoa, cv. Criollo) (Argout *et al.* 2011), and *Camellia sinensis* (tea, cv. Yunkang 10) (Xia *et al.* 2017) have also been sequenced. A combination of an increasing bioinformatic capability and the decreasing cost of sequencing has increased research teams' capability to predict the key regulatory genes in various metabolic pathways, providing the foundation for GE to modify the associated traits. Another exciting development is the proliferation of genome editing tools, especially the development of the CRISPR/Cas9 system and its successful demonstration in poplar (Jinek *et al.* 2012; Zhou *et al.* 2015; Elorriaga *et al.* 2018). Advances have also been made in overcoming the recalcitrance in tissue culture and transformation, particularly in angiosperm tree species. This review will summarize some of the recent progress in forest biotechnology in relation to the capacity for GE and gene editing, and then discuss opportunities and obstacles to its commercial application.

## Growth and yield

Growth improvements in forestry essentially fall into the categories of yield potential (greater growth under non-stressful conditions) and yield preservation (adequate growth, or at least survival, under stressful conditions). Breeding has also been directed at improving the traits of trees in ways that might not necessarily enhance the productivity in terms of tons of wood produced per hectare but increase the amount or value of marketable product (*e.g.*, stand uniformity, stem straightness, and reduced forking). An analysis of gains resulting from breeding in an agricultural crop, soybean, concluded that increases in productivity over the past 80 yr were primarily achieved through improving processes that affect yield potential: increased light interception, increased conversion of energy to biomass (*i.e.*, carbon fixation), and improved allocation of biomass to the harvested portion of the plant (Koester *et al.* 2014). It seems likely that improvements in yield for trees will fit similar categories as for crops but differ in preference for energy allocation to woody biomass in the main stem rather than seed production.

Although it is certain that tree breeding will continue to make improvements in yield, the potential for transformation to add genes that supplement what is available in the native genomes is enticing. Several years ago, Dubouzet *et al.* (2013) reviewed many possible strategies to increase tree biomass using transgenes, which included manipulation of phytohormone pathways to alter tree architecture, improved uptake and utilization of water and nutrients, modifications to photosynthesis and carbon utilization, and improved tolerance of biotic and abiotic stresses. Relatively few genes have undergone testing in tree species.

## Regulatory pathways

Manipulation of the biosynthesis and response pathways for several plant hormones has altered growth in ways that suggest an increase in biomass can be achieved. The most fully investigated example in forest trees was where increased internode length and growth could be seen when a gibberellic acid (GA) biosynthetic enzyme, GA 20-oxidase (GA20ox), was overexpressed in hybrid poplar (Eriksson *et al.* 2000). In contrast, an enzyme that deactivates GA, GA 2-oxidase, had the opposite effect on growth (Gou *et al.* 2011; Elias *et al.* 2012). One challenge in the strong upregulation of GA 20-oxidase using the CaMV 35S promoter was that it was associated with reduced adventitious root formation (Eriksson *et al.* 2000; Niu *et al.* 2013). Because rooted cuttings are the method of propagation for elite genotypes of many forest tree species, there is the likelihood that production could become less efficient or costs could increase when methods are adjusted to accommodate the GA phenotypes. Furthermore, the phenotype suggests the possibility that some of the enhancement in stem biomass is a result of allocation of energy that would ordinarily be used in root growth, a potentially risky strategy for a crop growing multiple years in a field environment.

To investigate whether more tightly regulated modification of GA synthesis might mitigate some of these effects, several transformation experiments in hybrid poplar tested GA20ox driven by a range of promoters. One construct used *P. trichocarpa* GA20ox7 expressed by its native promoter, which is expected to be comparable to an increase in copy number for an endogenous gene (Han *et al.* 2011; Lu *et al.* 2015). Other constructs included a different *P. trichocarpa* isoform of GA20ox driven by various *Populus* promoters preferentially expressed in shoot tips, internodes, or roots (Lu *et al.* 2015) and a pine GA20ox driven by the promoter from a *Populus* fasciclin-like gene (*DX15*) expressed in developing xylem (Jeon *et al.* 2016). Each of these constructs led to a reduction of the abnormal phenotype associated with high GA production and improved growth in greenhouse experiments when the promoter was *PtGA2ox*, *PtRGL1-1*(*REPRESSOR OF GAL3-LIKE*), *PtGA20ox7* (Lu *et al.*

2015), or *DX15* (Jeon *et al.* 2016). However, when the first two of these were subjected to a second, larger test in the greenhouse, the growth effect was not confirmed. When tested in the field, only the *RGL1-1* promoter construct provided a statistically significant growth improvement (Lu *et al.* 2015).

Recent studies have also evaluated the role of brassinosteroid biosynthesis on growth. Increased brassinosteroid synthesis has resulted in increased vegetative and seed yields in several herbaceous species (Choe *et al.* 2001; Sahni *et al.* 2016) but has only recently been examined in woody species. *PtCYP85A3*, the putative *Populus* ortholog of the *Arabidopsis thaliana* cytochrome P450 that catalyzes the final step in brassinolide biosynthesis, was tested in hybrid poplar *Populus trichocarpa*. Strong expression of this gene using the CaMV 35S promoter led to statistically significant increased growth in greenhouse and field tests (Jin *et al.* 2017). After 2 yr of growth in the field, the best performing line had a height 50% greater than the controls and a diameter more than 30% greater. Multiple secondary cell wall-associated genes (*PtMYB2*, *PtMYB18*, *PtMYB20*, *PtCesA5*, and *PtCesA17*) were found to be upregulated in the stems of the PtCYP85A3 overexpressing plants.

A relatively new avenue for increasing wood production might be found in members of the CLAVATA-LIKE family of peptide ligands and their receptors. In *A. thaliana*, *CLE41* encodes the precursor for a 12-amino-acid regulatory peptide known as tracheary element differentiation inhibitory factor (TDIF), whose receptor is encoded by *PXY* (reviewed in Etchells *et al.* 2015). Mutation of *PXY* caused production of abnormal vascular bundles, while constitutive overexpression of *CLE41* led to loss of apical dominance and increased vascular development (Etchells and Turner 2010). The *Populus* orthologs of these genes were cloned from and tested in hybrid poplar; overexpression increased wood formation under greenhouse conditions and gave normal form, but only when tissue-specific promoters were used (Etchells *et al.* 2015). The promoter from the cambium-expressed *Populus* homolog of *AINTEGUMENTA* was used with the *PttPXY* coding region, and the phloem-expressed *PttPP2* promoter was used with *PttCLE41*. The structure of the wood formed appeared normal, with increased stem diameter that resulted from more cells. Increased height growth was associated with larger leaves and formation of more internodes.

## Other genes tested in tree species

The processes of lignin formation and photorespiration release copious amounts of free ammonia and reassimilation of this nitrogen is essential for efficient growth. It has been estimated that, for herbaceous crops, up to 5% of shoot nitrogen can be lost as $NH_3$ during the growing season (Matsson and Schjoerring 1997). Under conditions of abundant nitrogen, the efficiency of recycling this $NH_3$ might not have a large effect on yield, but under typical field conditions, it is plausible that there would be a potential benefit. Various isoforms of glutamine synthetase (GS) perform the primary role in recovery of ammonia byproduct from photorespiration and lignin biosynthesis (Wallsgrove *et al.* 1987; Suárez *et al.* 2002). In hybrid poplar, constitutive expression of a pine GS1a gene was associated with increased growth under both greenhouse and field conditions (Gallardo *et al.* 1999; Jing *et al.* 2004; Coleman *et al.* 2012).

Some genes that have been tested in *Populus deltoides* to determine their effects on cell wall composition also exhibited increased growth. For example, an RNAi construct directed against a galacturonosyl transferase homolog (homologous to *A. thaliana GAUT12*) produced gains of 17–38% for shoot dry weight in a greenhouse trial (Biswal *et al.* 2015).

An additional pool of potential growth enhancing genes can be found in patents and patent applications. The latter in particular may have been filed on preliminary results and should be considered speculative. Tuskan and Kalluri (2016) described how RNAi directed against a *Populus* IDQ signaling protein led to increased biomass. Kirst (2014) described a poplar gene that contains a conserved DUF3339 domain, of interest because it was associated with improved growth in a (*P. trichocarpa* × *P. deltoides*) × *P. deltoides* pseudo-backcross. Expression of a *P. trichocarpa* allele of this gene in transgenic *Populus tremula* × *Populus alba* was found to cause increased size of vessel elements in the xylem, improved hydraulic conductivity, and increased growth rates under greenhouse conditions.

Researchers at SweTree Technologies have disclosed sequences from poplars that potentially can improve yield based on greenhouse experiments with transformed trees. Hertzberg *et al.* (2015a) were issued a patent with claims for improving growth *via* overexpression of a histone lysine methyltransferase, which provided an 11% increase in average diameter of transgenic lines as compared to the average of the control trees. A second patent claims reduced expression of a TRAF-like protein, a putative ubiquitinyl hydrolase, which produced a 24% increase in plant height relative to controls (Hertzberg *et al.* 2015b).

Another application describes enhancement of light capture by expression of protein chromophores, such as green fluorescent protein. The preferred chromophore would include a chloroplast targeting peptide and an excitation frequency in the yellow-green color range, where chlorophyll has an absorption minimum. The application reports increased growth for transgenic *Eucalyptus* expressing mCherry (Siegel *et al.* 2013).

The examples provided above still need validation through extensive field testing, and it may be that few of them end up providing a commercially valuable benefit. To guard against this, additional candidates should be pursued. Genome-wide

association studies, such as those underway in *Populus* (McKown *et al.* 2014), are a means of identifying genes and alleles that contribute to increased biomass and thus may be suitable targets for investigation.

There are also multiple genes that have provided evidence of enhanced biomass production in GE plants using herbaceous species (Lima *et al.* 2017). Improved growth was achieved in tobacco (*Nicotiana tabacum*) *via* reduction of nonphotochemical quenching by expressing a combination of violaxanthin de-epoxidase, zeaxanthin epoxidase, and exogenous photosystem II subunit S (Kromdijk *et al.* 2016). Increased expression of combinations of several genes involved in carbon flux, sedoheptulose-1,7-bisphosphatase (SBPase), fructose-1,6-bisphosphate aldolase (FBPA), a subunit of glycine decarboxylase (GDC-H), and a chloroplast-targeted inorganic carbon transporter (*ictB*) from cyanobacterium *Synechococcus* also yielded increased growth in transgenic *A. thaliana* or tobacco (Simkin *et al.* 2015, 2017). The glutamine synthetase pathway for ammonia reassimilation includes glutamine oxoglutarate aminotransferase (GOGAT, also known as glutamate synthase), glutamate dehydrogenase (GDH), and isocitrate dehydrogenase, which are further potential targets for manipulation (Suárez *et al.* 2002; McAllister *et al.* 2012).

Beyond these examples, the developing field of synthetic biology offers possibilities for more dramatic improvements in light capture, light energy conversion, and carbon fixation (Schwander *et al.* 2016). Potential avenues of research include altering the light harvesting machinery to make use of near infrared wavelengths, reengineering ribulose-1,5-bisphosphate carboxylase, and devising new, more efficient pathways for recycling the products of photorespiration (discussed in Ort *et al.* 2015; Betti *et al.* 2016).

## Cell wall and wood properties

The economic value of woody biomass is inherently associated with the biochemical components of the cell wall, which are important materials supporting commodity products, chemicals, energy, and other byproducts. Thus, there has been a strong interest in modifying cell wall traits to enhance secondary xylem formation or improve ease of use of the components. The two predominant cell wall components, cellulose and lignin, have been researched for decades. Hemicelluloses, the third major type of polymer, have also been the subject of GE studies, as have cell wall-associated pectins. On a molecular level, most of the studies have focused on ways to improve cellulose content or the efficiency of extracting it from its association with lignin. Additionally, conversion of biopolymers from feedstocks into biofuels has been studied; these biopolymers offer a promising and sustainable alternative to traditional petroleum-derived energy. Tables 1 and 2 summarize the recent transgenic studies in tree species on genetic modification of cell wall polysaccharides and lignin, respectively.

## Cellulose

Cellulose, the most abundant biopolymer in the world, represents approximately one-half of the woody biomass produced by trees and is composed of individual (1–4) β-linked D-glucan chains bound together to form highly ordered semi-crystalline microfibril bundles, which serve as a scaffold around which other cell wall polymers assemble (Taylor *et al.* 1992; Mellerowicz *et al.* 2001). Cellulose in secondary cell walls plays a fundamental role in preserving the structural integrity required for vertical growth in trees. Cellulose is believed to be typically synthesized by enzyme complexes composed of subunits encoded by three co-expressed genes, with different isoforms of the subunits used during production of primary and secondary cell walls (Kumar and Turner 2015). Repressing the expression of any one of the secondary cell wall-specific cellulose synthase genes negatively impacts cellulose deposition. For example, silencing of a *PtdCesA8* transgene in *Populus tremuloides* substantially reduced cellulose content, resulting in collapsed xylem vessels and stunted vertical growth (Joshi *et al.* 2011).

Endoglucanases have been shown to play a key role in the ordered deposition of cellulose in primary cell walls (Nicol *et al.* 1998; Sato *et al.* 2001). Studies investigating the misregulation of KORRIGAN-like genes in trees have provided insight into their role in secondary cell wall cellulose deposition. RNAi suppression of various tree orthologs of KORRIGAN genes in GE white spruce (*P. glauca*), poplar (*P. trichocarpa*), and hybrid poplar have resulted in reductions in cellulose content, and in the case of angiosperms irregular xylem tissue (Maloney and Mansfield 2010; Maloney *et al.* 2012; Yu *et al.* 2013, 2014). In hybrid poplar, RNAi suppression of a KORRIGAN-like gene was reported to not only reduce cellulose deposition, but to increase cellulose crystallinity and decrease microfibril angle compared to wild-type (Maloney and Mansfield 2010). This work indicates that KOR-like genes influence not only cellulose deposition, but the ultrastructure of cellulose in secondary cell walls.

Researchers looking to modify cellulose in trees have also focused on altering carbon allocation to the cellulose synthase complex (CSC) by modifying the metabolic pathways that lead to the production of UDP-glucose, the immediate biosynthetic precursor to cellulose. One study used UDP-glucose pyrophosphorylase (UGPase), which catalyzes the conversion of glucose-1-phosphate to UDP-glucose (Kleczkowski 1994). Poplar overexpressing a UGPase from *Acetobacter xylinum* displayed a substantial increase in cellulose content, but also exhibited a

**Table 1** Examples of genetic modifications made to cell wall components in trees, with emphasis on hemicellulose, pectin, and cellulose

| Cell wall component | Gene function | Gene expression change | Transformant species | References |
|---|---|---|---|---|
| Hemicellulose | Glycosyl transferase | *PoTG47C* ↓ | *Populus alba* × *P. tremula* | Lee *et al.* (2009) |
| | Glycosyl transferase | *PtrGT8D1/2* ↓ | *Populus trichocarpa* | Li *et al.* (2011a, b) |
| | Endotransglycosylase | *PtxtXyn10A* ↓ | *Populus tremula* × *P. tremuloides* | Derba-Maceluch *et al.* (2015) |
| | Glucuronxylan methyl transferase | *PtrDUF579-3* ↓ | *Populus* × *P. euramericana* | Song *et al.* (2016) |
| Pectin | Pectin methyl transferases | *PttPME1* ↑↓ | *Populus tremula* × *P. tremuloides* | Siedlecka *et al.* (2008) |
| | Pectate lyase | *PtxtPL1-27* ↑ | *Populus tremula* × *P. tremuloides* | Biswal *et al.* (2014) |
| | Galacturonosyl transferase | *PdGAUT12* ↓ | *Populus deltoides* | Biswal *et al.* (2015) |
| Cellulose | Cellulose synthase | *PtdCesA8* ↓ | *Populus tremuloides* | Joshi *et al.* (2011) |
| | Endoglucanase | *PaxgKOR* ↓ | *Populus alba* × *P. grandidentata* | Maloney and Mansfield (2010) |
| | Endoglucanase | *PgKOR* ↓ | *Picea glauca* | Maloney *et al.* (2012) |
| | Endoglucanase | *PtKOR1* ↓ | *Populus trichocarpa* | Yu *et al.* (2014) |
| | Endoglucanase | *PtrCel9A6* ↓ | *Populus trichocarpa* | Yu *et al.* (2013) |
| | Sucrose synthase | *PttSUS1*, *PttSUS2* ↓ | *Populus tremula* × *P. tremuloides* | Gerber *et al.* (2014) |
| | Fructokinase | *FRK2* ↓ | *Populus tremula* × *P. tremuloides* | Roach *et al.* (2012) |
| | UDPase | *Acetobacter xylinum* derived *UGPase* ↑ | *Populus alba* × *P. grandidentata* | Coleman *et al.* (2007) |
| | Sucrose synthase | *Gossypium hirsutum* derived *SuSy* ↑ | *Populus alba* × *P. grandidentata* | Coleman *et al.* (2009) |
| | Sucrose-6-phosphate synthase and sucrose-6-phosphatase | *Arabidopsis thaliana*-derived *SPS-SPP fusion construct* ↑ | *Populus alba* × *P. grandidentata* | Maloney *et al.* (2015) |

Up-arrow shows expression increase, down-arrow shows expression decrease that resulted

slight reduction in growth potential (Coleman *et al.* 2007). A second study tested overexpression of a cotton sucrose synthase (SuSy), which catalyzes the formation of UDP-glucose and fructose from sucrose. This led to more crystalline cell walls, enhanced wood density, and thicker cell walls without impacting plant growth and development in poplar (Coleman *et al.* 2009). In both cases, introduction of *SuSy* and *UGPase* transgenes resulted in an increase in the soluble sugars present in leaf tissue, indicating that altering the availability of UDP-glucose influences sink strength in trees (Coleman *et al.* 2007, 2009). Poplar relies on a passive mechanism to move photoassimilate from source to sink tissues (Turgeon and Medville 1998) and increasing available UDP-glucose promotes the reallocation of carbon skeletons toward cell wall deposition, thereby reducing the soluble sugars present in sink cells and promoting movement of photoassimilates toward sink tissue. Subsequent findings showed that RNAi suppression of two endogenous *SuSy* genes in hybrid poplar resulted in no significant changes in relative composition of cellulose, lignin, and hemicellulose; however, wood density was substantially decreased (Gerber *et al.* 2014). Suppression of these genes also resulted in a reduction in the available pools of hexose phosphates, providing further support that in poplar *SuSy* genes play a role in regulating carbon partitioning to wood cell walls (Gerber *et al.* 2014).

There is also evidence that further upstream modification of sucrose biosynthesis in trees can influence xylem density and wood properties. Sucrose is synthesized in source tissues from UDP-glucoase and fructose-6-phosphate *via* a two-step process by sucrose-6-phosphate synthase (SPS) and sucrose-6-phosphatase (SPP) before being transported to sink tissues. Hybrid poplar overexpressing an *A. thaliana SPS-SPP* fusion construct displayed an increase in wood density, an increase in soluble sugars within source leaves, and an increase in overall growth characteristics (Maloney *et al.* 2015).

Once arriving in sink tissues, sucrose enters metabolism by being broken down either into UDP-glucose and fructose, or into glucose and fructose, which can be phosphorylated before being metabolized for use in cell wall biosynthesis (Sturm and Tang 1999). RNAi suppression of fructokinases (FRKs) in hybrid poplar (*P. tremula* × *P. tremuloides*) resulted in thinner cell walls and a reduction in cellulose, indicating that FRK can also influence carbon partitioning to cell wall biosynthesis (Roach *et al.* 2012). Taken together, these studies strongly suggest that

**Table 2** Examples of genetic modification of cell wall genes within the lignin biosynthetic pathway of tree species

| Genetic modification | Gene function | Gene expression change | Transformant species | References |
|---|---|---|---|---|
| Homologous expression | Cinnamate 4-hydroxylase | *C4H* ↓ | *Populus tremula* × *P. tremuloides* | Bjurhager *et al.* (2010) |
| | 4-Coumarate:coenzyme A (CoA) ligase | *4CL* ↓ | *Populus trichocarpa* | Hu *et al.* (1999); Schmidt *et al.* (2009) |
| | 4-Coumarate:CoA ligase | *4CL* ↓ | *Populus tremula* × *P. alba* | Voelker *et al.* (2011a, b) |
| | 4-Coumarate:CoA ligase | *4CL* ↓ | *Populus tomentosa* | Tian *et al.* (2013) |
| | 4-Coumarate:CoA ligase | *4CL* ↓ | *Populus tomentosa* | Tian *et al.* (2013) |
| | 4-Coumarate:CoA ligase | *4CL* ↓ | *Populus tomentosa* | Jia *et al.* (2004) |
| | 4-Coumarate:CoA ligase | *4CL* ↓ | *Populus tremuloides* | Li *et al.* (2003) |
| | 4-Coumarate:CoA ligase | *4CL* ↓ | *Pinus radiata* | Wagner *et al.* (2009) |
| | 4-Coumarate:CoA ligase | *4CL* ↓ | *Betula pendula* | Sutela *et al.* (2014) |
| | Hydroxycinnamoyl-CoA shikimate hydroxycinnamoyltransferase | *HCT* ↓ | *Pinus radiata* | Wagner *et al.* (2007) |
| | *p*-Coumaroyl-CoA 3-hydroxylase | *C3'H* ↓ | *Populus grandidentata* × *P. alba* | Coleman *et al.* (2008a); Ralph *et al.* (2012) |
| | *p*-Coumaroyl-CoA 3-hydroxylase | *C3'H* ↓ | *Eucalyptus urophylla* × *E. grandis* | Sykes *et al.* (2015) |
| | Cinnamoyl-CoA reductase | *CCR* ↓ | *Populus trichocarpa* | Leplé *et al.* (2007) |
| | Cinnamoyl-CoA reductase | *CCR* ↓ | *Pinus radiata* | Wagner *et al.* (2007, 2013) |
| | Cinnamoyl-CoA reductase | *CCR* ↓ | *Picea abies* | Wadenbäck *et al.* (2008) |
| | Ferulate/coniferaldehyde 5-hydroxylase | *F5H* ↑ | *Populus tremula* × *P. alba* | Franke *et al.* (2000); Huntley *et al.* (2003); Stewart *et al.* (2009) |
| | Caffeic acid O-methyltransferase | *COMT* ↓ | *Populus trichocarpa* × *P. deltoides* | Van Doorsselaere *et al.* (1995); Lu *et al.* (2010) |
| | Caffeic acid O-methyltransferase | *COMT* ↓ | *Populus tremula* × *P. alba* | Marita *et al.* (2001) |
| | Caffeoyl-CoA O-methyltransferase | *CCoAOMT* ↓ | *Populus tremula* × *P. alba* | Meyermans *et al.* (2000) |
| | Caffeoyl-CoA O-methyltransferase | *CCoAOMT* ↓ | *Pinus radiata* | Wagner *et al.* (2011) |
| | Peroxidase | *PtrPO21* ↓ | *Populus trichocarpa* | Lin *et al.* (2016) |
| | MicroRNA | *Ptr-miR397a* ↑ | *Populus trichocarpa* | Lu *et al.* (2013) |
| Heterologous expression | Feruloyl-CoA monolignol transferase | *Angelica sinensis*-derived FMT | *Populus tremuloides* | Wilkerson *et al.* (2014) |
| | p-Coumaroyl-CoA monolignol transferase | *Oryza sativa*-derived PMT | *Populus trichocarpa* | Smith *et al.* (2015) |
| | Tyrosine cell wall peptide | *Petroselinum crispum*-derived TYR | *Populus nigra* × *P. maximowiczii* | Liang *et al.* (2008) |
| | Coniferaldehyde 5-hydroxylase | *Liquidambar styraciflua*-derived CAld5H | *Populus tremuloides* | Li *et al.* (2003) |
| | Glutamine synthase | *Pinus pinaster*-derived GS1a | *Populus tremula* × *P. alba* | Gallardo *et al.* (1999); Coleman *et al.* (2012) |
| | MicroRNA | *Zea mays*-derived miRCG1↑ | *Populus tremula* × *P. alba* | Rubinelli *et al.* (2013) |
| Multigene manipulation | Coniferaldehyde 5-hydroxylase: 4-coumarate:coenzyme A ligase | *Liquidambar styraciflua*-derived CAld5H ↑: 4CL ↓ | *Populus tremuloides* | Li *et al.* (2003); Suzuki *et al.* (2010) |
| | Coniferaldehyde 5-hydroxylase: 4-coumarate: coenzyme A ligase | *Liquidambar styraciflua*-derived CAld5H ↑: 4CL ↓ | *Populus trichocarpa* | Xiang *et al.* (2015) |
| | Ferulate/coniferaldehyde 5-hydroxylase: 4-coumarate:coenzyme A ligase | F5H ↓: 4CL ↑ | *Populus nigra* × *P. maximowiczii* | Min *et al.* (2013, 2014a, b) |
| | Ferulate/coniferaldehyde 5-hydroxylase: caffeic acid O-methyltransferase | *Liquidambar styraciflua*-derived F5H ↑: *L. styraciflua* derived COMT ↑ | *Pinus radiata* | Wagner *et al.* (2015) |

Up-arrow shows expression increase, *down-arrow* shows expression decrease that resulted

altering availability of soluble carbohydrates in source and sink tissues can serve as a useful method of modifying cellulose production in trees.

## Hemicellulose

Hemicelluloses are a heterogeneous group of branched polysaccharides that associate with both cellulose microfibrils and lignin. In dicot angiosperms such as poplars and eucalypts, hemicelluloses are composed predominantly of glucuronoxylan (GX) chains. These are xylan chains that are partially substituted by glucuronic acid and 4-O-methyl-glucuronic acid (GlcA and MeGlcA, respectively) groups (Timell 1967). In conifers, the predominant hemicelluloses are glucomannan and glucuronoarabinoxylan (Pettersen 1984). These polymers can also be decorated with acetyl groups. Biosynthesis of GX polymers involves several different classes of Golgi-targeted glycosyl transferases that produce the primary backbone, side chain, and reducing end sequence (Rennie and Scheller 2014). During the biochemical conversion of wood biomass, hemicellulose content and composition are factors limiting saccharification, because they limit the access of cellulases to cellulose (Himmel *et al.* 2007). RNAi suppression of glucuronoxylan glycosyl transferase genes in hybrid poplar (*P. tremula* × *P. alba)* resulted in marked decreases in glucuronoxylan content, as well as improved hydrolysis by cellulases (Lee *et al.* 2009). RNAi suppression of two other putative xylan glycosyl transferases in *P. trichocarpa* also resulted in drastically reduced xylan content (Li *et al.* 2011a, b). However, in both studies, the reduction in cell wall xylan resulted in a corresponding decrease of secondary cell wall thickness, collapsed vessel elements, and a reduction in stem modulus of elasticity (Lee *et al.* 2009; Li *et al.* 2011a, b).

Similarly, augmentation of xylan-modifying enzymes in poplar has been shown to impact cell wall digestibility or ease of hydrolysis. RNAi suppression of *PtrDUF579-3*, an enzyme that mediates the methylation of the GlcA groups associated with GX chains, resulted in a reduction in GlcA and methylated GlcA. Genetically engineered lines subjected to acid pretreatment and cellulase digestion released greater quantities of xylose and glucose compared to wild-type trees (Song *et al.* 2016). MeGlcA side residues have been suggested to interact with lignin through covalent ester linkages (Takahashi and Koshijima 1988). Suppression of *PtrDUF579*-3 appears to improve cellulase access to cellulose by altering the linkages between the major cell wall polymers (Song *et al.* 2016).

Xylan-modifying enzymes also influence the overall secondary cell wall structure. Suppression of a xylan endotransglycosylase, *PtxtXyn10A*, in hybrid poplar (*P. tremula* × *P. tremuloides*) resulted in reduction in the microfibril angle (Derba-Maceluch *et al.* 2015). The authors suggest that *PtxtXyn10A* may serve to release tensional stresses during the deposition of secondary cell walls, and suppression results in a build-up of stress leading to re-orientation of cortical microtubules which direct the deposition of cellulose during cell wall development (Derba-Maceluch *et al.* 2015).

## Pectin

Pectins are a group of heterogeneous polysaccharides rich in galacturonic acid. In wood cell walls, pectins are composed mainly of homogalacturonan (HG) and rhamnogalacturonan I (RGI), which form into a pectic-matrix surrounding the cellulosic-glycan network (Willats *et al.* 2001). The final structure of the pectin network is decided by a suite of transglycosylases, esterases, hydrolases, and pectate lyases located in the cell wall (Fry 2004).

Pectins are a major component of primary cell walls but are largely absent in secondary cell walls (Jarvis 1984). Although secondary cell walls form the bulk of woody biomass, there is evidence that modification of pectin may alter saccharification yields in trees. For example, overexpression of a pectate lyase gene in hybrid poplar (*P. tremula* × *P. tremuloides*) increased the solubility of cell wall pectins and xylans, and improved yields during enzymatic saccharification (Biswal *et al.* 2014). Additionally, RNAi suppression of a cottonwood (*P. deltoides*) glycosyltransferase gene, orthologous to a galacturonosyltransferase involved in biosynthesis of pectic HG in *A. thaliana*, *r*esulted in a reduction in both hemicellulose and pectin, and produced trees with more easily extractable cell walls (Biswal *et al.* 2015).

Pectin methylesterases (PMEs) de-esterify HG chains, facilitating intramolecular linkages within the pectin network (Pelloux *et al.* 2007). Overexpression of a PME in hybrid poplar (*P. tremula* × *P. tremuloides*) was shown to decrease the degree of esterification in HG chains, while RNAi suppression of the same PME resulted a reduction in total HG content, likely because of the increased susceptibility of highly methyl-esterified HG to degradation (Siedlecka *et al.* 2008).

## Lignin

Lignin, the second most abundant biopolymer, is often considered the glue that cements the other secondary cell wall components together. In doing so, it provides structural stability to the secondary cell wall while also aiding in water conduction and plant defense. At a superficial level, lignin is comprised of varying ratios of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) moieties polymerized within the cell wall (Ralph *et al.* 2004). In angiosperm trees, half or more of lignin may be derived from S units, while in conifers only H and G moieties are found. Closer inspection reveals that there is vast diversity within the polymer composition (Morreel *et al.*

2004; Ralph *et al.* 2004; Mottiar *et al.* 2016). For example, 5-hydroxyconiferyl alcohol, hydroxycinnamaldehydes, and hydroxycinnamic acids, which are products of incomplete monolignol biosynthesis, have all been clearly found within the lignin polymer (Vanholme *et al.* 2008). Additionally, atypical monolignol conjugates like sinapyl *p*-hydroxybenzoate and coniferyl *p*-coumarate, among others, have been found incorporated in the polymer, yielding great physicochemical diversity (Morreel *et al.* 2004; Ralph *et al.* 2004; Del Rio *et al.* 2007).

Delignification typically relies on the addition of acid or alkali at high temperatures to remove lignin for fiber separation in pulp and paper production and to facilitate the saccharification of lignocellulosic material for biofuel production (Mansfield 2009). As the lignin biosynthetic pathway has been largely mapped and provides biosynthetic mechanisms explaining some of the diversity found within the polymer (Humphreys and Chapple 2002), researchers have focused on manipulating various steps in the pathway to engineer lignin and its constitutive monomers to improve digestibility. Myriad potential solutions to the lignin challenge have been proposed, but most notable efforts have focused on reducing the lignin content in plants by downregulating critical lignin biosynthetic genes. These studies are summarized in Table 2, where the genes are ordered to reflect the progress of the steps in monolignol biosynthesis. For example, cinnamate 4-hydroxylase (C4H) catalyzes the first oxidation of the aromatic ring, and acts in the formation of all three lignin subunit types. Downregulation of this gene in hybrid poplar (*P. tremula* × *P. tremuloides*) resulted in a 30% reduction in lignin content (Bjurhager *et al.* 2010). Marked reduction in lignin content has also been achieved with the downregulation of 4-coumarate:coenzyme A ligase (4CL). Some GE lines showed as much as 45% reduction in total lignin and a significant increase in the presence of *p*-hydroxycinnamic acids, such as *p*-coumaric, caffeic, sinapic, and ferulic acid (Hu *et al.* 1999; Li *et al.* 2003; Jia *et al.* 2004; Schmidt *et al.* 2009; Wagner *et al.* 2009; Voelker *et al.* 2011a; Tian *et al.* 2013; Sutela *et al.* 2014).

Downregulation of a putative hydroxycinnamoyltransferase (HCT), a major artery enzyme that directs substrate toward S and G monolignol biosynthesis, was shown to be an effective target for reducing lignin content in tissue-cultured tracheary elements of *Pinus radiata*, where a 42% reduction in lignin content was observed (Wagner *et al.* 2007). Next downstream in the pathway, *p*-coumaroyl-CoA 3′-hydroxylase (C3'H) has been downregulated in hybrid poplar (*Populus grandidentata* × *P. alba*) and *Eucalyptus* (*E. urophylla* × *E. grandis*), with the result of significant reductions of lignin content, from a conventional 22 and 29.6% to as low as 10 and 21.7% total cell wall content, respectively (Coleman *et al.* 2008a; Sykes *et al.* 2015). Further analysis of the poplar GE lines revealed alteration in the H:S:G ratios within the polymer, with the most significant change being an increase in the H:G ratio (Coleman *et al.* 2008a; Ralph *et al.* 2012). Suppression of C3'H in hybrid poplar also resulted in irregular and small vessel elements (Fig. 1).

Downregulation of cinnamoyl-CoA reductase (CCR), the penultimate step in monolignol biosynthesis, resulted in a 50% reduction in lignin content in *P. trichocarpa*, 46% reduction in *P. radiata*, and an 8% reduction in *P. abies* (Leplé *et al.* 2007; Wadenbäck *et al.* 2008; Wagner *et al.* 2013). Interestingly, downregulation of CCR also resulted in the lignin being significantly altered in S:G ratio and a dramatic shift in the metabolite profile, with increased levels of *p*-coumaryl, caffeic acid, and ferulic acid hexoses (Wagner *et al.* 2013).

Although these GE lines do, on average, have reduced lignin and presumably an increased ease of utilization, dramatic changes in lignin content can lead to significant alterations in the morphology and agronomic potential of plants (Coleman *et*

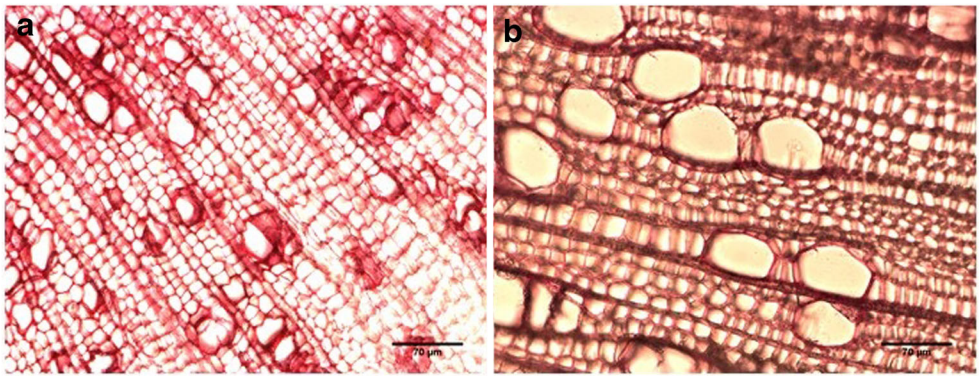


**Fig. 1** Microtome cross-sections of transgenic RNAi-mediated suppressed C3'H (*a*) and wild-type (*b*) hybrid poplar (*P. alba* x *grandidentata*) histochemically stained with Mäule's reagent. The brighter red intensity apparent in *panel A* is indicative of an enrichment in syringyl monomers in the lignin polymer. Also note the irregular and small vessel element in the transgenic xylem (*a*) compared to the wild-type xylem (*b*) that manifests from the overall reduction in total secondary cell wall lignin content due to C3'H suppression and overall perturbation of the lignin biosynthetic pathway.

*al.* 2009; Min *et al.* 2012; Sykes *et al.* 2015). Trees with reduced lignin content tend to lack structural integrity, and have stunted growth, reduced water conductivity and vascular stability, and an increased vulnerability to pathogens and pests (Coleman *et al.* 2008b; Voelker *et al.* 2011a, b; Bonawitz and Chapple 2013). As a result, recent efforts have focused more on altering the physiochemical properties of lignin without significantly altering its content or functionality.

One such target has been altering the ratio of lignin monomers. The H:G:S ratio varies considerably from species to species, and greatly affects the processability of xylem fibers and vessel elements. For example, S-rich lignins, which are found in angiosperm species, contain more β–O–4 bonds compared to G-rich lignins, which are seen in gymnosperm species and contain more β–5, 5–5, and β–β carbon–carbon bonds. The higher instance of β-ethers common to S-rich lignin make it more susceptible to alkali-mediated degradation, and thus increasing the S:G ratio has been a major scientific pursuit within the last few decades (Mansfield *et al.* 2012).

The expression levels of ferulate/coniferaldehyde-5-hydroxylase (F5H or Cald5H), caffeic acid O-methyltransferase (COMT), caffeoyl-coenzyme A *O*-methyltransferase (CCoAOMT), and MIR156 family microRNA (miRCg1) have all been shown to impact the S:G ratio. For example, the S:G ratios in hybrid poplar (*P. tremula* × *P. alba*) and *P. tremuloides* were increased with the overexpression of F5H, resulting in an increase of S units within the polymer to as high as 97.5%, compared to the innate 68% (Franke *et al.* 2000; Huntley *et al.* 2003; Li *et al.* 2003; Stewart *et al.* 2009). Li *et al.* (2003) also combined upregulating F5H with downregulating 4CL in *P. tremuloides* and found that the combination simultaneously increased the S:G ratio, reduced lignin content, altered the phenolic composition, and improved the enzyme-mediated saccharification yields (Suzuki *et al.* 2010; Xiang *et al.* 2015). Although increasing the abundance of S units in hybrid poplar has met with great success, the alteration of the S:G ratio in S-deficient conifer species has not been reported for intact trees. However, a successful proof of principle was achieved with simultaneous expression of *Liquidambar styraciflua* F5H and COMT in *P. radiata* tracheary element cell cultures (Wagner *et al.* 2015).

Downregulation of COMT in poplars (*P. trichocarpa* × *P. deltoides* and *P. tremula* × *P. alba*) resulted in a significant decrease in S units, an increased number of β–O–4 bonds in G and S end units, and integration of 5-hydroxyconiferyl alcohol within the polymer (Van Doorsselaere *et al.* 1995; Marita *et al.* 2001; Lu *et al.* 2010). Downregulation of CCoAOMT, while resulting in a decrease in the overall lignin content, also led to a significant increase in the S:G ratio of poplar (*P. tremula* × *P. alba*) and an increase in the H:G ratio in *P. radiata* (Meyermans *et al.* 2000; Wagner *et al.* 2011). In addition to reducing lignin content and increasing the H:G ratio in *P. radiata*, downregulation of CCoAOMT resulted in the novel integration of caffeoyl alcohol into the lignin polymer (Wagner *et al.* 2011).

Although there are many ways in which the phenylpropanoid pathway can be modified to reduce lignin content, some researchers have looked outside of the common lignin biosynthetic genes for a solution. For example, Lin *et al.* (2016) identified and downregulated a class III peroxidase in *P. trichocarpa* (*PtrPO21*) which resulted in an average lignin reduction of 20%. This decline in lignin was attributed to a decrease in reducing enzymes, therefore decreasing the degree of radicalization of monolignols, and thus polymerization. A similar result was observed with the upregulation of *Ptr-miR397a*, a microRNA that targets laccases (Lu *et al.* 2013). Moreover, expression of the maize miRCg1 in hybrid poplar (*P. tremula* × *P. alba*) resulted in a 30% reduction in lignin and a significantly lower S:G ratio (Rubinelli *et al.* 2013).

These significant studies have revealed the inherent plasticity of lignification, in terms of both the biosynthetic pathway and the structure of the lignin polymer itself (Vanholme *et al.* 2008; Stewart *et al.* 2009; Eudes *et al.* 2012; Mottiar *et al.* 2016). Based on this malleability, there are ongoing efforts to develop novel lignin with altered content and composition to improve digestibility or utility (Weng *et al.* 2008; Vanholme *et al.* 2012). One tack has been to identify genes that can contribute to the synthesis of favorable monolignol conjugates, which, when integrated into the lignin backbone, increase the number of alkali-cleavable bonds and improve delignification rates (Wilkerson *et al.* 2014). These more easily hydrolyzable lignins are also referred to as "zip-lignins." One method to increase the number of alkali-cleavable bonds is through the introduction of esters into the lignin polymer. Acylated monolignols are produced naturally in many plants by acyltransferases acting prior to the oxidation and radical coupling of lignin monomers (Del Rio *et al.* 2007). To-date, only four such enzymes, dubbed monolignol transferases, have been identified. Two *p*-coumaroyl-CoA:monolignol transferases (PMTs) in *Oryza sativa* and *Brachypodium distachyon* catalyze the conjugation of *p*-coumaroyl moieties to monolignols, and two feruloyl-CoA:monolignol transferases (FMTs) in *Angelica sinensis* and *O. sativa* conjugate feruloyl moieties to coniferyl and sinapyl alcohol (Withers *et al.* 2012; Petrik *et al.* 2014; Wilkerson *et al.* 2014; Karlen *et al.* 2016). Expression of an *A. sinensis* FMT in hybrid poplar (*P. alba* × *P. grandidentata*) achieved the integration of ferulate–monolignol conjugates into the lignin (Wilkerson *et al.* 2014), and the novel ester bonds within feruloylated lignin units resulted in increased efficiency of delignification. This approach was also shown to be successful with the expression of PMT from *O. sativa* in hybrid poplar, resulting in *p*-coumarate conjugates incorporated into lignin (Smith *et al.* 2015). Introducing or increasing the abundance of these

hydroxycinnamates (*p*-coumarate, ferulate, and sinapate) is one area of particular interest in the pursuit of zip-lignin (Ralph 2010).

An alternative approach to zip-lignins is to increase the occurrence of peptide cross-linkages within the lignin polymer to increase the sites for protease action. This was achieved with the overexpression of a transgene encoding a high tyrosine-content peptide in hybrid poplar (*P. deltoides* × *P. nigra*), which resulted in lignin that showed increased susceptibility to enzyme-mediated digestion and had increased polysaccharide yields post-treatment (Liang *et al.* 2008).

In addition, lignin has been altered by reduced carbohydrate coupling. Reducing the linkages between lignin and carbohydrate secondary cell wall components limits the need for intensive pre-saccharification treatment by increasing carbohydrate accessibility for enzyme-mediated saccharification. Min *et al.* (2013) observed that with F5H upregulation and 4CL downregulation in hybrid poplar (*P. nigra* × *P. maximowiczii*), there was an increase in the lignin carbohydrate cross links within the secondary cell wall, which resulted in less accessible cellulose for saccharification (Min *et al.* 2014a, b). Moreover, Coleman *et al.* (2012) observed an increase in the S:G ratio in the lignin of hybrid poplar (*P. tremula* × *P. alba*) in which a pine cytosolic glutamine synthetase was expressed (Gallardo *et al.* 1999). Notably, these GE events also had significantly elevated levels of secondary cell wall carbohydrates including glucose, galactose, xylose, and mannose.

The many examples provided demonstrate that lignin with altered content and composition can readily be achieved through modifying expression of genes in the phenylpropanoid pathway. However, obtaining a commercially useful result will probably require modification of multiple steps in the network of biosynthetic reactions, and there is a limitation in the number of traits that feasibly can be manipulated through multigene alteration. The genetic manipulation of the transcriptional regulators controlling suites of genes involved in secondary cell wall development may provide a means for altering more complex wood phenotypes. For example, manipulation of the transcriptional regulator ARBORKNOX2 (ARK2), a homeobox gene in hybrid poplar (*P. tremula* × *P. tremuloides*) resulted in changes to lignin and cellulose content that were consistent with simultaneous transcriptional changes in key genes involved in both lignin and cellulose biosynthesis (Du *et al.* 2009). This approach was also shown to be effective with the overexpression of MYB transcription factors in *P. glauca* (white spruce), and NAC domain transcription factors in *Eucalyptus* (Bomal *et al.* 2008; Hussey *et al.* 2011). Meanwhile, single nucleotide polymorphisms were examined in *P. trichocarpa* to identify genes associated with physical and chemical characteristics of the secondary cell wall (Porth *et al.* 2013). In another approach, hierarchical gene regulatory network models were developed to identify regulatory genes based on RNA-seq analysis of poplar xylem protoplasts overexpressing NAC domain transcription factors (Lin *et al.* 2013). The progress in understanding the regulation of cell wall components will help to design the wood properties to suit for efficient and sustainable utilization while maintaining growth and stress resistance characteristics.

## Biotic stress resistance

Biotic stress can significantly impact tree health and survival, reducing forest productivity. However, the means to counter major disease or pest threats is very limited for most tree species. The US Forest Service publishes a summary of the most important insect and disease conditions threatening forests (Karel and Man 2017). Each of these causes significant damage each year. For example, the mountain pine beetle (*Dendroctonus ponderosae*) epidemic killed about 4 million hectares of pine forest in the western states of the USA in 2009, while the current epidemic in Western Canada has been estimated to have affected more than 18 million hectares of pine forest and continues to spread eastward (Cooke and Carroll 2017). According to a US Forest Service report, bark beetle species have damaged more than 16 million hectares of conifer trees stands in the western USA from 1997 to 2011 (Unired States Forest Service 2011). Other pests such as gypsy moth (*Lymantria dispar*), southern pine beetle (*Dendroctonus frontalis*), emerald ash borer (*Agrilus planipennis*), sudden oak death (*Phytophthora ramorum*), and fusiform rust (*Cronartium quercuum* f. sp. *fusiform*.) are killing or damaging millions of hectares of conifers and angiosperm trees each year. Evidence shows that the outbreaks are associated with the changes in environmental conditions (Anacker *et al.* 2008). Breeding for resistance is limited by the long flowering age for many tree species, especially for the economically important conifers (Johnson 1939; Castellanos-Hernández *et al.* 2011). Even though genetic control of fusiform resistance has been identified in loblolly pine (Wilcox *et al.* 1996; Amerson *et al.* 2015), the application of molecular tools has been very limited due to a lack of precision and cost. The main practice to reduce the impact of rust has been screening for resistance at seedling stage and the use of resistant genetic materials in loblolly and slash pine seed orchards (Schmidt 2003; Cowling and Young 2013).

In comparison to forest trees, GE applications in horticultural trees are more advanced, though its commercial uptake has been limited as well (Table 3). The earliest success was in response to papaya ring spot virus, which nearly devastated Hawaii's papaya industry. Researchers at Cornell University, in conjunction with Hawaiian papaya growers, developed a transgenic, resistant papaya using parasite-derived resistance (PDR) in the early 1990s (Ferreira *et al.* 2002), and gained federal approval for commercial orchards in Hawaii in 1998

**Table 3** Examples of genetic modification for improved pest resistance in tree species

| Tree species | Pest | Genetic modification or technique | Gene | Regulatory status (if known) | References |
|---|---|---|---|---|---|
| Pathogens | | | | | |
| *Carica papaya* | Papaya ringspot virus | Parasite-derived resistance (PDR) | Papaya ringspot virus coat protein | Approved by USDA and FDA in 1997 | Gonsalves (1998) |
| *Castanea dentata* | Chestnut blight (*Cryphonectria parasitica*) | Heterologous expression | Oxalate oxidase | Application to US federal agencies currently in process | Newhouse *et al.* (2014) |
| *Castanea sativa* | Chestnut blight (*Cryphonectria parasitica*) | Homologous expression | CsCh3 chitinase ↑ | NA | Corredoira *et al.* (2016) |
| *Citrus sinensis* 'Hamlin' | Multiple bacterial and viral disease | Homologous expression | Salicylic acid-binding protein 2 (SABP2) ↑ | NA | Attílio *et al.* (2014) |
| *Citrus sinensis* | Citrus canker (*Xanthomonas citri* subsp. *citri* (Xcc)) | CRISPR/Cas9/sgRNA-mediated gene editing | Lateral Organ Boundaries 1 (CsLOB1) promoter | NA | Peng *et al.* (2017) |
| *Citrus × paradisi* | Citrus canker (*Xanthomonas citri* subsp. *citri* (Xcc)) | CRISPR/Cas9/sgRNA-mediated gene editing | Lateral Organ Boundaries 1 (CsLOB1) | NA | Jia *et al.* (2016, 2017) |
| *Citrus × paradisi* | Citrus tristeza virus | Heterologous expression | Untranslatable coat protein gene (uncp) and *Galanthus nivalis* agglutinin (gna) | NA | Yang *et al.* (2000); Stover *et al.* (2013) |
| *Eucalyptus urophylla* | *Pseudomonas solanacearum* | Heterologous expression | Cecropin d | NA | Shao *et al.* (2002) |
| *Juglans regia* | *Agrobacterium tumefaciens* | RNAi | *iaa*M, *ipt* | NA | Walawage *et al.* (2013) |
| *Picea abies* | *Ceratocystis polonica* fungus | Homologous expression | *Picea abies* leucoanthocyanidin reductase (PaLAR3) ↑ | NA | Hammerbacher *et al.* (2014) |
| *Populus* hybrid | *Septoria musiva* fungus | Heterologous expression | Oxalate oxidase | NA | Liang *et al.* (2001) |
| *Prunus domestica* | Plum pox virus | RNAi | Plum pox virus coat protein | Approved in US, not commercially planted | Scorza *et al.* (2013, 2016) |
| *Prunus* rootstock cultivars 'Gisela 6' and 'Gisela 7' | *Prunus* necrotic ring spot virus | RNAi | A 2.5-kb IR of the coding sequence targeted at six *Prunus* viruses | NA | Song *et al.* (2013) |
| *Theobroma cacao* | Oomycetes (*Phytophthora*, *Colletotrichum*) | Heterologous expression | Secretory PI3P-binding proteins | NA | Helliwell *et al.* (2016) |
| *Ulmus americana* | Dutch elm disease (*Ophiostoma* spp) | Heterologous expression | Synthetic antimicrobial peptide ESF39A | No application in progress | Newhouse *et al.* (2007) |
| Herbivorous pests | | | | | |
| *Diospyros kaki* | Lepidopteran insects | Heterologous expression | Bt *CryIAc* | NA | Tao *et al.* (1997); Dandekar *et al.* (2002) |
| *Eucalyptus camaldulensis* | Chrysomelid beetles | Heterologous expression | Bt *Cry3Aa* | NA | Harcourt *et al.* (2000) |
| *Juglans regia* | Codling moth (*Cydia pomonella*) | Heterologous expression | Bt *CryIAc* | NA | Dandekar *et al.* (1998) |
| *Juglans regia* | Root lesion nematode (*Pratylenchus vulnus*) | RNAi | Pv010 (putative mRNA processing subunit) | NA | Walawage *et al.* (2013) |
| NA | Asian longhorn beetle (*Anoplophora glabripennis*) | NA | Bt *Cry3Aa* fused to a cellulase-binding peptide | NA | Guo *et al.* (2012) |
| *Picea abies* | Herbivorous insects and fungal pathogens | Homologous expression | Isoprenyl diphosphate synthase (PaIDS) ↑ | NA | Schmidt *et al.* (2010) |
| *Picea glauca* | Spruce budworm (*Choristoneura fumiferana*) | Heterologous expression | Bt *Cry1Ab* | NA | Lachance *et al.* (2007) |

**Table 3** (continued)

| Tree species | Pest | Genetic modification or technique | Gene | Regulatory status (if known) | References |
|---|---|---|---|---|---|
| *Pinus radiata* | Painted apple moth (*Teia anartoides*) | Heterologous expression | Bt *Cry1Ac* | NA | Grace *et al.* (2005) |
| *Populus* hybrid | Lepidopteran insects | Homologous expression | MYB134 tannin regulatory gene ↑ | NA | Mellway *et al.* (2009); Boeckler *et al.* (2014) |
| *Populus* hybrid clone 741 | Multiple Lepidopteran defoliating insects | Heterologous expression | Bt *Cry1Ac* and API | Commercially grown in China since 2002 | Tian *et al.* (2000); FAO (2010) |
| *Populus nigra* | Multiple Lepidopteran defoliating insects | Heterologous expression | Bt *Cry1Aa* | Commercially grown in China since 2002 | Hu *et al.* (2001) |
| *Prunus serotina* | Cambial mining insects | Homologous expression | Prunasin hydrolase isoform 3 (PH3) ↑ and mandelonitrile lyase isoform 4 (MDL4) ↑ | NA | Wang and Pijut (2014b) |
| *Tsuga canadensis* and *Tsuga caroliniana* | Hemlock wooly adelgid (*Adelges tsugae*) | Somatic embryogenesis, cryopreservation and propagation of resistant lines | NA | NA | Merkle *et al.* (2014) |

(Suzuki *et al.* 2007). A genetically engineered plum (*Prunus domestica* L.) was developed soon after, using post-transcriptional gene silencing to confer resistance to plum pox virus (Scorza *et al.* 2013). To date, this "C5" plum has been cleared by federal regulators for cultivation only in the USA (Scorza *et al.* 2016), but is not currently used in commercial production.

A more recent disease threatening the entire citrus industry is huánglóngbìng (HLB), or citrus greening, which can affect all citrus cultivars, stunting trees and rendering the fruit unmarketable. Transgenic citrus trees have been developed in an attempt to provide disease resistance to the bacteria that causes HLB (Dutt *et al.* 2008, 2015; Hajeri *et al.* 2014) or to the insect vector (Hajeri *et al.* 2014). The trees developed by Dr. Eric Mirkov's team at Texas A&M University are overexpressing a defense gene originated from spinach, which encodes a protein identified as being toxic to a variety of bacteria and fungi, including the canker causing *Candidatus liberibacter asiaticus* (Voosen 2014). *The trees* have gone through multi-year trials and have shown good resistance phenotypes. The Southern Gardens Citrus Corporation has taken steps to deregulate the transgenic HLB-resistant citrus.

Citrus canker is another bacterial disease affecting the citrus industry. An exciting development has been reported recently using CRISPR/Cas9 technology to edit the susceptibility gene *CsLOB1*, a member of the Lateral Organ Boundaries Domain (LBD) gene family of plant transcription factors (Jia *et al.* 2017). By knocking out both alleles of *CsLOB1* in the highly susceptible grapefruit cultivar Duncan (*Citrus paradisi*), two transgenic lines were produced which showed enhanced resistance. These trees did not develop any canker symptoms upon inoculation with *Xanthomonas citri* ssp. *citri* (Xcc). The engineered varieties do not have any introduced foreign genes, which could potentially reduce some of the regulatory burden in the USA.

A novel approach to fungal and oomycete resistance was developed in *T. cacao* using phosphatidylinositol-3-phosphate (PI3P) binding proteins (Helliwell *et al.* 2016). These proteins bind to pathogen effectors and block their entry into plant cells. Resistance conferred with PI3P-binding proteins may be more stable than that based on host resistance (R) proteins, as PI3P-binding proteins target a greater diversity of pathogen effectors (Helliwell *et al.* 2016). Transgenic *T. cacao* trees secreting PI3P-binding proteins showed enhanced resistance to *Phytophthora tropicalis*, *P. palmivora*, and the fungal pathogen *Colletotrichum theobromicola*.

For forest trees, China was the first country where transgenic trees were released for commercial use. Insect-resistant poplar trees containing a modified *Cry1Ac* toxin gene from *Bt* were approved and released for planting in 2002 (Food and Agricutural Organization 2010). By 2015, approximately 543 ha of *Bt* poplar were planted in China (James 2015). The released events were crossed with *P. deltoides*, and the

2-yr-old hybrids showed excellent efficacy against *L. dispar* larvae feeding on leaves (Hu *et al.* 2014). To date, no significant negative environmental impacts caused by the transgenic poplars have been reported (Hu *et al.* 2014).

*Cry* genes have also been used for transformation of hybrid poplar (Klocko *et al.* 2013), *Eucalyptus* (Harcourt *et al.* 2000), Monterey pine (Grace *et al.* 2005), and walnut (Dandekar *et al.* 1998). In field studies of poplar transformed with a Coleopteran-active *Cry3a Bt*-toxin gene, there was approximately 14% growth advantage, even in years of slight damage by the cottonwood leaf beetle (Klocko *et al.* 2013). *Cry* genes tagged with a DNA fragment that encodes a cellulase-binding peptide showed increased lethality to Asian longhorn beetle larvae, attributable to increased toxin retention in the midgut as a result of the protein binding to Cx-cellulase produced by the larvae (Guo *et al.* 2012). Transgenic American elm (*Ulmus americana*) has been developed with a synthetic antimicrobial peptide, conferring some resistance to Dutch elm disease (Newhouse *et al.* 2007). Black cherry (*Prunus serotina*) has been transformed with genes coding for prunasin hydrolase and mandelonitrile lyase to enhance hydrogen cyanide (HCN) production to increase resistance to cambial-mining insects (Wang and Pijut 2014b). Black cherry naturally produces HCN, and the researchers used endogenous genes with alternative promoters for enhanced enzymatic production. The efficacy of this method is still under investigation.

Research using GE and transcriptomics has allowed for greater understanding of existing plant defenses to biotic stress. Economically important spruce species have been the subject of several such investigations. The transcriptomes of white spruce (*P. glauca*) cells were described at baseline levels and under methyl jasmonate-induced conditions, differentiated by cell type (cortical resin duct cells, phenolic cells, and phloem) through the use of laser microdissection (Celedon *et al.* 2017). Another study shed light on genes involved in the biosynthesis of stone cells in Sitka spruce (*Picea sitchensis*), which serve as a physical defense against stem-boring insects (bark beetles and weevils) in conifers (Whitehill *et al.* 2016). Bark beetles can be vectors of fungal pathogens, and an increase in specific proanthocyanidins in spruce bark after fungal infection was shown to be important to pathogenesis. Genes involved in this response were investigated in a bacterial system and by overexpression in transgenic Norway spruce (*P. abies*) (Hammerbacher *et al.* 2014). Such basic research is critical for identifying candidate genes for the next generation of biotech trees.

## Chestnut blight resistance

In eastern North America, American chestnut (*Castanea dentata*) was one of the most prevalent trees in the deciduous forests prior to the twentieth century, valued for its nut crop and rot-resistant wood. Chestnut blight, a canker disease caused by the fungal pathogen *Cryphonectria parasitica*, spread through the species' range in the decades following its introduction from Asia around 1904, destroying billions of chestnut trees (Anagnostakis 1987). Conventional breeding programs have been conducted to introduce the resistant genes from Chinese chestnut (*Castanea mollissima*) since the 1920s. One such program by The American Chestnut Foundation (TACF) is still active (Anagnostakis 2012; Steiner *et al.* 2016). The TACF breeding program has begun to implement genomic selection to improve the speed and efficiency of candidate selection (Steiner *et al.* 2016). Blight resistance in American chestnut was also attempted by mutagenesis, or "mutational breeding" with no success (Dietz 1978; Burnworth 2002; Liang 2011). The best results from GE approaches have been from the State University of New York's College of Environmental Science and Forestry and collaborating institutions. They developed a transformation system (Merkle *et al.* 1991; Carraway *et al.* 1994; Polin *et al.* 2006), and have tested many candidate genes for enhancing chestnut blight resistance or tolerance. American chestnut has proved to be a difficult species; these techniques have taken decades to develop.

The most promising blight tolerance gene was chosen based on observations of variation in fungal virulence between strains. Virulent strains of *C. parasitica* produce far greater quantities of oxalate than hypovirulent strains (Havir and Anagnostakis 1983). The detoxification of oxalate might thus provide resistance to chestnut blight. Oxalate oxidase (OxO), which catalyzes the degradation of oxalate into $H_2O_2$ and $CO_2$, is produced in a wide variety of plants including wheat (*Triticum aestivum*) and other cereal grains (Lane 1994). An early test showed that transgenic hybrid poplar expressing the OxO transgene showed increased tolerance to oxalic acid and resistance to the fungal pathogen *Septoria musiva* (Liang *et al.* 2001). When transgenic American chestnut trees were later developed with the OxO gene driven by the CaMV 35S promoter, they demonstrated resistance to chestnut blight better than American chestnut controls; in some cases, better even than blight resistant Chinese chestnut controls (Zhang *et al.* 2013; Newhouse *et al.* 2014; Fig. 2). A petition to the USDA for deregulation of the best performing event is in preparation; researchers are also working with the EPA and FDA toward the use of these trees for horticultural and restoration plantings. American chestnut transformed with the OxO gene might have an advantage in durability of the resistance, as OxO is not lethal to the blight fungus. Instead, OxO mitigates the fungus' ability to kill living plant tissue, while still allowing the fungus to live as a saprophyte. Chestnut blight also lives as a saprophyte on other species such


**Fig. 2** Transgenic American chestnut expressing the oxalate oxidase (OxO) gene. (*a*) Transgenic American chestnut trees in the New York Botanical Garden, near to where the blight was first discovered in 1904. (*b*) Bumblebee on an American chestnut catkin (male flower). Studies were done on the ecological effects of OxO-producing chestnut on species including bumblebees. (*c*) Leaf disk assay (green living tissue, brown dead tissue) demonstrating oxalic acid tolerance lowest in (*1*) American chestnut, higher in (*2*) Chinese chestnut, and highest in (*3*) transgenic American chestnut. (*d*), (*e*) Siblings from transgenic American chestnut and infected with blight, (*d*) did not inherit the OxO gene and is susceptible, (*e*) inherited the OxO gene and is resistant.

as oak (*Quercus* spp.), maple (*Acer rubrum*), hickory (*Carya ovata*), and sumac (*Rhus typhina*) (Stipes *et al.* 1978; Baird 1991; Davis *et al.* 1997; Frigimelica and Faccoli 1999), which serve as refugia. This will likely increase sustainability of the tolerance mechanism by reducing selection of any fungal mutations that would overcome the effects of the OxO.

## Abiotic stress resistance

In addition to the biotic challenges, tree growth and health are profoundly influenced by environmental factors such as water availability, nutrients, and temperature. There are multiple ways for GE to help trees cope with abiotic stresses to preserve biomass growth. Globally, climate change brings more

unpredictable and severe weather patterns that are not only having a negative impact on biomass accumulation but also pose a threat to the survival of trees. In many arid areas, limited water availability and salinization of soils retards tree growth or increases tree mortality. For these reasons, improving drought or salt tolerance has been an important part of research programs. For example, traditionally in northern China, poplar varieties have been planted in large areas as windbreaks, environmental protection belts, or to harvest wood for various purposes. Stunted growth and a high mortality rate in areas with frequent drought or salt stress are key limiting factors affecting productivity. A salt-tolerant poplar (*Populus* × *xiaozhannica* cv. "Balizuangyang") containing a mannitol dehydrogenase gene (*mtl*D) from *Escherichia coli* has been developed and permitted for large plantings in China (Liu *et al.* 2000; Sun *et al.* 2002).

There have also been other promising results from GE of abiotic stress responses. Genetically engineered hybrid poplar trees expressing genes to increase the accumulation of osmolytes or molecular chaperones to protect the cellular components under drought conditions exhibited improved growth under stress (Takabe *et al.* 2008; Li *et al.* 2011a, b). The greenhouse survival of Pinus virginiana transgenic plants expressing an ERF/AP2 transcription factor from pepper was much higher than controls when exposed to 48°C (Tang *et al.* 2005). Abiotic stresses can lead to accumulation of reactive oxygen species (ROS) damaging cells. Resistance to such oxidative stresses was successfully demonstrated in greenhouse-grown *Populus alba* × *Poplus glandulosa* by inducible expression of AtNDPK2 (*A. thaliana* nucleoside diphosphate kinase driven by a sweet potato anionic peroxidase promoter) or *Populus sieboldii* × *Populus grandidentata* by constitutively expressing a horseradish peroxidase (CaMV35S::prxC1a) (Kawaoka *et al.* 2003; Kim *et al.* 2011). There have been a number of other promising developments in abiotic stress tolerance in plants that await evaluation in trees (*e.g.*, Dai *et al.* 2007; Yang *et al.* 2012; Baldoni *et al.* 2015; Butt *et al.* 2017; Zhang *et al.* 2017).

A good example of engineering abiotic stress resistance is the development of freeze tolerant *Eucalyptus* (Hinchee *et al.* 2011). The target tree was an elite clone of *E. grandis* × *E. urophylla*, which exhibited fast growth, superior fiber characteristics, and high pulp yield, but was sensitive to winter freezes in the Southeastern USA. The concept was to provide a source of competitive and high-quality fiber in the USA by extending the growing range from southern Florida to northern Florida and the coastal region of the southern USA. The elite clone was transformed with a cassette that contained the *A. thaliana* cold-inducible promoter *rd29A* (Yamaguchi-Shinozaki and Shinozaki 1993) driving CBF2, a member of the CBF/DREB (C-repeat dehydration-responsive element binding) transcription factor family and a regulator of the cold-response pathway in *A. thaliana* (Jaglo-Ottosen *et al.* 1998; Liu *et al.* 1998). The cassette also included a pollen control gene to mitigate potential gene flow concerns (Hinchee *et al.* 2009, 2011; Zhang *et al.* 2012a). Single insertion events were tested in 21 replicated field trials across eight different locations representing USDA Hardiness Zones 8a (potential kill zone), 8b (target zone), and 9a (freeze stress-free zone) (Hinchee *et al.* 2011). The top events showed comparable growth to non-transgenic controls in areas with mild or no freezing during the winter months. In the target zone, with the temperature as low as − 8.4°C during the fifth winter season, the best-performing line suffered only a 10% dieback as compared to 99% dieback for the controls. The top two best performing events were submitted in petition 11-019-01p (United States Department of Agriculture Animal and Plant Health Inspection Service 2013 and links therein) for deregulation. However, the decision on the petition is still pending (United States Department of Agriculture Animal and Plant Health Inspection Service 2017), and the company does not appear to have released information to the public on whether the cold tolerance has remained effective over time, including during the unusually cold temperatures observed in the Southern USA in recent years.

Recently, progress has been made in understanding cold stress signaling and regulation pathways in model plants. For instance, a report showed that a transcription factor gene, *BZR1*, a key component of the brassinosteroid signaling pathway, acts upstream of the *CBF* pathway, and can potentially be a target for regulating cold tolerance (Li *et al.* 2017). Other transcription factors such as ICE1 (a basic-helix-loop-helix type transcription factor) or CAMTAs (calmodulin-binding transcription activators) were shown to upregulate CBFs (Chinnusamy *et al.* 2003; Doherty *et al.* 2009). More recently, protein kinase OST1 (OPEN STOMATA1), also known as SnRK2.6, was found to phosphorylate ICE1 enhancing its activation of *CBF3* expression under cold stress (Ding *et al.* 2015). Overexpression of ICE1 and OST1 enhanced freezing tolerance in *A. thaliana* (Ding *et al.* 2015); overexpression of ICE1 alone improved rice survival and led to accumulation of proline under cold stress (Xiang *et al.* 2008). However, these genes have yet to be tested in forest trees.

## Reproduction control

**Accelerated flowering** Tree domestication and breeding has been hindered by the prolonged juvenile period of tree species. Gymnosperms, such as *Picea* and *Pinus*, can take more than 15 yr from seed or juvenile propagules to flower (Häggman *et al.* 2016). Even for fast-growing angiosperm species, such as poplars and *Eucalyptus*, it generally takes more than 3 yr to flower and sometimes much longer. Estimates vary widely, however, depending on genotype,

environment, and the application of floral stimulating treatments (Klocko *et al.* 2016a; McGarry *et al.* 2017).

With the goal of shortened breeding cycles, early onset of sexual reproduction has been a focus of reproductive modification studies in trees. Traditionally, early flowering can be induced by grafting flowering twigs onto rootstocks, applying chemical (*e.g.*, fertilizer and hormone), environmental treatments such as vernalization and photoperiod manipulation, and physical treatments such as stem girdling and root pruning (Flachowsky *et al.* 2009; McGarry *et al.* 2017). These traditional methods, however, are costly, take time to be optimized, and are often too slow or ineffective (McGarry *et al.* 2017).

Recent discoveries of the genes that control flowering have transformed the landscape for control of floral initiation and structure (reviewed by Krizek and Fletcher 2005; Chandler 2012; Ó'Maoiléidigh *et al.* 2014), and enabled GE-based floral induction. The first success in a tree was reported in poplar, where the floral meristem identity gene *LEAFY* (*LFY*) from *A. thaliana* was overexpressed under the control of the 35S promoter (Weigel and Nilsson 1995). Later studies with *LFY* and its orthologs, however, often found low precocious flowering rate and abnormal floral morphology (Rottmann *et al.* 2000; Flachowsky *et al.* 2009). More effective floral initiation has been achieved with the *FLOWERING LOCUS T* (*FT*) gene and the *TERMINAL FLOWER1* (*TFL1*) gene—two homologous genes that act distinctly in the floral initiation pathway. The *FT* gene encodes the long-distance florigenic signal in *A. thaliana*, promotes the transition from vegetative to reproductive growth, and has been found to be functionally conserved across diverse plant species (Böhlenius *et al.* 2006; Lifschitz *et al.* 2006; Corbesier *et al.* 2007; Lin *et al.* 2007; Klintenäs *et al.* 2012). Constitutive overexpression of the *FT* gene and its orthologs has successfully induced early flowering in poplar (Zhang *et al.* 2010), *Eucalyptus* (Klocko *et al.* 2016a), and several fruit trees such as apple (*Malus* × *domestica* Borkh.; Yamagishi *et al.* 2014), citrus (*Citrus excelsa*; Velázquez *et al.* 2016), plum (Srinivasan *et al.* 2012), and olive (*Olea europaea* L.; Haberman *et al.* 2017), usually without strong alterations in floral morphology or fertility. However, poplar requires special conditions for *FT* to induce viable pollen or seed (Zhang *et al.* 2010; Hoenicka *et al.* 2016). Alternatively, precocious flowering can be conferred by suppression of the reproductive transition inhibitor gene *TFL1*, as reported in poplar, apple, and pear (*Pyrus communis* L.; Mohamed *et al.* 2010; Flachowsky *et al.* 2012; Freiman *et al.* 2012; Yamagishi *et al.* 2014, 2016). Several other floral genes, such as *BpMADS4* (a flowering-associated MADS box gene from silver birch; *Betula pendula* Roth.), *FLOWERING PROMOTING FACTOR1* (*FPF1*) and *FRUITFUL* (*FUL*) from *A. thaliana* have also been found to be effective in promoting precocious flowering in some plant species (Kania *et al.* 1997; Teper-Bamnolker and Samach 2005; Flachowsky *et al.* 2007; Weigl *et al.* 2015; Häggman *et al.* 2016).

## Suppressed reproduction

Because many commercially important species, such as poplars and *Eucalyptus*, are primarily vegetatively propagated, delayed flowering and sexual sterility can be beneficial in tree breeding. Male-sterility can help in producing hybrids, and reduction or complete loss of fertility is likely to promote allocation of photosynthetic resources to vegetative growth (Strauss *et al.* 1995) and therefore may improve wood yield. Delayed flowering can be achieved by mutation or suppression of genes that promote floral initiation with RNAi or gene editing technology. Flowering can also be postponed or prevented by overexpression of flowering suppressors or modified activators (reviewed in Brunner *et al.* 2007 and Vining *et al.* 2015; Fig. 3). Additionally, the juvenile-to-adult transition in trees has been shown to be controlled by miRNA. A prolonged juvenile phase has been observed in *Populus* overexpressing miR156—a master regulator of vegetative phase change (Wang *et al.* 2011). Sexual sterility has also been sought as a major means to alleviate concerns over release of exotic and invasive species, as well as from transgene dispersal from GE trees. In addition to conventional polyploidization to produce highly sterile triploids and aneuploids (Lu *et al.* 2014; Shi *et al.* 2015, 2016; Tokumoto *et al.* 2016; Yang *et al.* 2016; Guo *et al.* 2017), GE-based genetic ablation (*i.e.*, elimination of floral organs) has been achieved by using a floral-specific promoter to drive the expression of a cytotoxic gene. For example, the *PrMC*2 promoter, a male-cone-specific promoter cloned from pine, and the *TA29* promoter, a tapetum-specific promoter cloned from tobacco, have been used to drive the expression of a barnase gene to produce stable, multi-year sterility in field-grown *Pinus*, *Eucalyptus*, and *Populus* trees (Zhang *et al.* 2012a; Elorriaga *et al.* 2014). Li *et al.* (2016b) created a chimeric promoter by fusing a reversed intron fragment of *PtAG2*, an *AGAMOUS* orthologue from *Populus trichocarpa*, with a short version of the 35S promoter. This chimeric promoter showed floral-specific activity in tobacco and was used to drive the expression of the *Diphtheria* toxin A (DT-A) in tobacco and poplar to produce flowerless plants (to date sterility has been demonstrated only in tobacco).

Disturbed expression of floral genes can also impart bisexual sterility. For example, RNA interference (RNAi) has been used to suppress the expression of the floral meristem identity gene *LFY* in poplar (Klocko *et al.* 2016b) and the floral homeotic C-class gene, *AGAMOUS* (*AG*) in apple (Klocko *et al.* 2016c). Although reduced fertility or complete sterility was observed in both cases, the efficiency was low and variable. Direct editing of floral genes using gene editing technology, which often


**Fig. 3** RNAi suppression of floral genes causes sterility in *Populus* sp. (*a*), (*b*) RNAi sterility trial in Corvallis, OR. (*c*) Wild-type control catkin in field. (*d*) RNAi-LFY/AG catkin, indicated by *arrow*, in field. (*e*) RNAi-AG catkin in field. (*f*) Wild-type control catkin under microscope. (*g*) RNAi-AG catkin under microscope.

leads to loss-of-function of the target gene, appears to be more stable for tree species with long life spans. In poplar, CRISPR-Cas nucleases have been used to target *LFY* and *AG* orthologs with an average mutation rate as high as 80% (Elorriaga *et al.* 2016, 2018). This is much more efficient than zinc-finger nucleases, which gave mutation rates of lower than 0.3% (Lu *et al.* 2016). Gene editing approaches could also be employed for repair of mutated sites, enabling continued breeding of elite, sterile lines where desired. With genome-wide transcriptomes accelerating the discovery of genes active during flowering in tree species (*e.g.*, Vining *et al.* 2015), many more

potential targets for gene editing will undoubtedly be identified in the future.

## Genome editing

Multiple gene editing technologies have been developed in the past two decades and continue to be improved rapidly. The overarching concept behind genome editing is that creating double-stranded breaks (DSBs) at targeted sites in the genome greatly increases the frequency of mutation and recombination at those sites. There are numerous reviews summarizing the early technologies to generate targeted DSBs using homing nucleases (Salomon and Puchta 1998), zinc finger nucleases (ZFNs; Kim *et al.* 1996), and transcription activator-like effector nucleases (TAL nucleases, also known as TALEN®; Li *et al.* 2010; Bogdanove and Voytas 2011). Zinc finger nucleases and TAL nucleases are similar in that they comprise a cluster of DNA binding domains fused to a nuclease. Methods using ZFNs or TAL nucleases entail identification of two binding sites, closely spaced and on opposite strands, and design and construction of two coding regions. The large size of vectors with two expression cassettes, combined with the repetitive nature of the coding sequences, means that there could be instability of the DNA in bacteria with resulting difficulties in transformation. One of the few tests of ZFN in a tree species, *Populus*, found that it caused a reduced rate of transformation and gave low rates of mutagenesis (Lu *et al.* 2015).

Most recently, the focus has been on the CRISPR/Cas9 system derived from an RNA-guided bacterial system for defense against viruses (Jinek *et al.* 2012). Here, the specificity of the Cas9 nuclease is directed by a synthetic single guide RNA (sgRNA). The sgRNA contains a segment of 20 nucleotides that can be designed complementary to a very wide range of target regions in the genome. Cas9 has a relatively large coding region (approximately 4 kb), but the fact that the sgRNAs are small (approximately 100 nt) and easily designed makes the system very convenient for targeting multiple sites simultaneously (Liang *et al.* 2017). Tests in poplars have shown that CRISPR/Cas9 cleavage and nonhomologous end joining (NHEJ) is very efficient, with most events having biallelic mutations of the targeted gene (Fan *et al.* 2015; Zhou *et al.* 2015; Elorriaga *et al.* 2018).

The CRISPR/Cas9 system is quickly becoming the most adopted tool for genome editing and further improvements are rapidly being made. As one example, mutations of Cas9 (Kleinstiver *et al.* 2015) and homologs of Cas9 from other species (Karvelis *et al.* 2015; Zetsche *et al.* 2015) have been identified with different requirements for binding sequence context, potentially allowing modification of a wider range of target sites. In another refinement, there have been recent demonstrations that Cas9 protein and sgRNA can be assembled into a ribonucleoprotein (RPN) complex and bombarded into cells of crops to achieve editing without Cas9 or guide DNA sequence integration (Svitashev *et al.* 2016; Liang *et al.* 2017). This can be very important for tree biotech where there is limited opportunity to use breeding to segregate the undesirable DNA sequences away.

The use of CRISPR/Cas9-based gene editing has many potential applications in biotech research on forest tree species. It should be possible to create biallelic knock-outs of almost any gene at a reasonably high efficiency (*e.g.*, Fan *et al.* 2015; Zhou *et al.* 2015), which are nearly essential for characterization of gene function in species that are long lived or cannot be easily self-pollinated. If it becomes possible to introduce the targeting sgRNAs using viral vectors (Ali *et al.* 2015), gene knock-out studies could be done even in species with low transformation efficiency. The previously mentioned elimination of the citrus genome binding site for a bacterially coded pathogenesis protein (Jia *et al.* 2017) and prevention of floral development (discussed previously) are other recent applications of gene editing in trees. However, more studies will be necessary to elucidate whether full or partial inactivation of the genes involved in wood structure and chemistry, such as those for lignin biosynthesis that have been widely targeted in gene suppression studies, would provide satisfactory wood modification and also normal tree growth and adaptability.

Upregulation ("knock-in") of a gene of interest through simple repair of DSBs seems plausible only in limited cases, such as by deletion of a known repressor binding site in a promoter or inactivation of a protein motif for rapid degradation (such as DELLA or a PEST motif, Rogers *et al.* 1986; Peng *et al.* 2017). In tomato (*Solanum lycopersicum* L.), the editing of selected repressor binding domains in meristem regulatory genes gave a wide variety of modifications to fruit characteristics (Rodríguez-Leal *et al.* 2017). In many cases, it may prove more practical to supplement the endogenous genes with an overexpression cassette through traditional transformation methods. In an extension of this, a widely expressed endogenous gene could be inactivated using a DSB followed by transformation with a replacement gene with a more specifically expressed promoter, analogous to using *c4h* mutant *A. thaliana* as a starting point to generate lines with vessel-specific expression of C4H activity (Yang *et al.* 2013).

The repair of DSBs through insertion of DNA *via* homologous recombination may provide some of the greatest opportunity for generating traits of value to forestry. Genes or gene segments can be swapped out for desired sequences by taking advantage of this mechanism. As can be seen from examples discussed in prior sections, commercially valuable traits can require modification or replacement of promoters (to increase expression or change an expression pattern) or protein coding sequences (for example, to enhance resistance to herbicides, insects, or pathogens). These can be achieved by generating a

DSB (or pair of DSBs) near the target region while simultaneously providing the desired sequence as a linear DNA fragment. This could also, of course, be used to modify an endogenous promoter to increase transcription within the normal context of gene expression, which should reduce unwanted pleiotropic effects.

Targeted production of DSBs also allows one to bias insertion events of transgenes so that they occur repeatedly at the same locus, even if only as a single insertion (hemizygosity). This should have great value as modified genes are used in breeding programs. If a given insertion event of an exogenous gene for disease resistance were to be successfully tested in one genetic background (such as the case of OxO in chestnut), it should be possible to introduce the same gene into the corresponding locus of unrelated genotypes by generating a DSB at that site—increasing confidence it will express properly. Such an outcome would then allow homozygous offspring to be produced in one generation after two genotypes with hemizygous insertions at the same locus are crossed. This would simplify inheritance and mitigate linkage drag and inbreeding depression that could otherwise result from repeated use of the original resistance event.

The major obstacle to gene replacement and targeted insertion is efficiency. For plant tissues other than cell suspensions, insertion of DNA *via* homologous recombination at DSBs does not occur very frequently. In *A. thaliana*, when the donor sequence was provided from a stably inserted transgene, the targeted insertion rate ranged from approximately 0.1 to 1% of the NHEJ rate (Fauser *et al.* 2012). It seems that gene replacement will require some sort of selection system such as an herbicide resistance gene within the donor sequence. If the selectable marker were flanked with short, unique, target sequences, it might then be efficiently removed by a further round of cleavage with Cas9 and the requisite sgRNA after useful lines have been identified.

## Transformation improvement

As more powerful molecular tools are becoming available for targeted gene identification and manipulation, the recalcitrance of many species and genotypes to transformation and/or regeneration looms as the major technical limitation to GE and gene editing. Although there has been significant progress with transformation of a few commercially important tree species, (reviews in Confalonieri *et al.* 2003; Merkle and Narin 2005; Nehra *et al.* 2005), most species and genotypes remain difficult, costly, or for practical purposes impossible to transform with current methods. Table 4 summarizes the recent progress in developing transformation capability with important angiosperm forest tree species.

A significant limitation to transformation is the ability to identify and regenerate transgenic cells (Maruyama and Hosoi 2015), whether it be through organogenesis or somatic embryogenesis (Campbell *et al.* 2003). Organogenesis systems have been the main method for GE of angiosperm forest species such as poplars and *Eucalyptus*. Somatic embryogenesis has been developed for most of the valuable conifers, such as some commercially important southern pine and spruce species, such as loblolly pine (*Pinus taeda*) and Norway spruce (*Picea abies*; see review Nehra *et al.* 2005). Somatic embryogenesis has also been the vehicle for clonal propagation and genetic modification for some angiosperm trees such as sweetgum and American chestnut, both of which have limited capability to regenerate *via* organogenesis (Merkle *et al.* 1991, 1998; Carraway *et al.* 1994; Merkle and Battle 2000; Nehra *et al.* 2005; Polin *et al.* 2006). Regeneration capacity is highly species- and genotype-dependent and is negatively associated with the maturity of trees from which the explants are collected (Merkle and Narin 2005; Castellanos-Hernández *et al.* 2011).

The regeneration bottleneck could be greatly reduced if transgenes could be deployed that would bestow regenerability on the transgenic cells, and this strategy appears to be the basis of a recent breakthrough in maize transformation. Lowe *et al.* (2016) demonstrated that by overexpressing the morphogenic genes *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*) from maize, they were able to increase transformation efficiency and enable transformation of previously extremely difficult genotypes. Moreover, they could use a wider variety of explants, found that high transformation efficiency could be achieved from experiments using mature corn seed or seedling leaf explants, and that the rate of embryogenic tissue growth was accelerated (Nardmann and Werr 2006; Lowe *et al.* 2016). Furthermore, the team tested and showed that the same technology worked well with sorghum, sugarcane, and rice—demonstrating it was truly a general rather, than a species- or genotype-dependent, innovation in monocots. Finally, because of the deleterious effects of the morphogenic genes on fertility, a drought-inducible recombinase system was used to excise the morphogenic genes during regeneration or propagation. The recombinase cassette effectively removed the morphogenic genes at a frequency of 60–89% of the single-copy T0 events.

This technology should be applicable to forest trees after adaptation and customization. The effectiveness of morphogenic genes on angiosperm plant species was first demonstrated in dicots, where somatic embryos were induced on various explants in *A. thaliana* and other species (Lotan *et al.* 1998; Boutilier *et al.* 2002; Lowe *et al.* 2002; Zuo *et al.* 2002; Stone *et al.* 2008), and these genes improved regeneration (Srinivasan *et al.* 2007; Deng *et al.* 2009). It is anticipated that such constructs optimized for angiosperms or conifers can directly be applied to embryogenesis systems using seeds or reproductive tissues as explants as was the case in monocots. Overexpression of a *Bbm* homolog isolated from *T. cacao* led

**Table 4** Examples of progress in genetic transformation methods for angiosperm tree species (since 2005)

| Transformant species | Explant | *Agrobacterium* strain**/plasmid/ | Results | References |
|---|---|---|---|---|
| *Betula pendula* | Leaf | C58C1, pGV3850, *uidA* | Gene transfer to early flowering genotypes | Lemmetyinen *et al.* (2008) |
| *B. pendula* | Callus and shoot culture | pBI121.1 with *uidA*, pRT99GUS with *uidA* and *nptII*, and pBI426 with *uidA*:*nptII*, *RbcS*, particle bombardment | Transient GUS expression, stable integration of *RbcS*; carbon use efficiency studied | Kontunen-Soppela *et al.* (2010) |
| *B. pendula* | Leaf | LBA4404, pAL4404, pBKL4KA, *Kit4*, *nptII*, *uidA* | Transgenic plants with pathogen resistance; Insect population and density in field trial; insect feeding bioassays; heterologous expression, ectomycorrhizal colonization | Vihervuori *et al.* (2008); Pasonen *et al.* (2009); Vihervuori *et al.* (2013) |
| *B. pendula* | Leaf | pHTT602, *BpMADS1*, *BpMADS6* | Changes in inflorescence and flower formation in transgenics | Lemmetyinen *et al.* (2008) |
| *B. pendula* | Leaf | C58C1, pGV2260, pBI121, *nptII*, *Bp4CL1* | Transgenic plants, soil microbiota studied | Seppänen *et al.* (2007) |
| *B. pendula* | Leaf | pBKL4K4, *chiIV*, *nptII* | Transgenic plants in field trial | Niskanen *et al.* (2011) |
| *B. platyphylla* | Leaf | LBA4404, *bgt*, *Bt*, *uidA* | Transgenic plants lethal to gypsy moth larvae; Transgene stability in long-term cultures | Zeng *et al.* (2009, 2010) |
| *B. platyphylla* | Leaf | LBA4404, pCAMBIA-2301, *nptII*, *uidA*, *bgt*, *Cry1A(b)* | Transgene expression stabile in long-term field trials | Zeng *et al.* (2011) |
| *B. platyphylla* | 4-week-old seedling | pBI121, pROKII-GFP, pROK-*BpCCR*, pFGC-*BpCCR* | Transient gene overexpression, gene silencing | Zhang *et al.* (2012b) |
| *B. platyphylla* | 3-week-old seedling | EHA105, pBI121 | Transient GUS expression | Zheng *et al.* (2012) |
| *B. platyphylla* | Leaf | EHA105, pROKii-MADS12, *BpMADS12* | No effect on flowering time, affected the number and size of female inflorescences, and decreased seed vigor | Li *et al.* (2016a) |
| *B. pubescens* | Leaf | CBE21, pBI121, pGS, *uidA*, *nptII*, *hpt*, *GS1* | Transgenic plants; lack of correlation between ammonium accumulation and survival | Lebedev *et al.* (2010, 2015) |
| *Broussonetia papyrifera* | Leaf and petiole | LBA4404, pCAMBIA 1301, *uidA*, *hpt*, *FaDREB2* | Transgenic plants; ectopic expression; osmotic tolerance | Li *et al.* (2008, 2011a, b) |
| *B. papyrifera* | Floral dip | P3301-121, *ppt*, *BpDREB2* | Transgenic expression in *Arabidopsis*, enhanced tolerance to salt and freezing | Sun *et al.* (2014) |
| *Carya illinoinensis* | Somatic embryo | EHA101/pCGN 7001, *nptII*, *uidA*; particle bombardment, pBI121.1, *uidA*, *nptII* | Transgenic plants; GUS expression, variation in gene expression, chimeric embryos | Vendrame and Wetzstein (2005) |
| *Castanea dentata* | Somatic embryo | EHA105, pVspB-Oxo, *OxO*, *bar*, *mgfp5-ER*; EHA105, pTACF3, pTACF7 | Regeneration of transformed embryos, transient *GFP* expression; transgenic plants; transgenic plants with enhanced pathogen—blight resistance, trait to T1 progeny | Polin *et al.* (2006); Maynard *et al.* (2006); Rothrock *et al.* (2007); Maynard *et al.* (2008); Zhang *et al.* (2013); Newhouse *et al.* (2014); Maynard *et al.* (2015) |
| *C. dentata* | Pollen | pBIN *35S-mgfp5-ER*, particle bombardment | Transient *GFP* expression | Fernando *et al.* (2006) |
| *C. dentata* | Callus | EHA105, pGPOxO | No change in cell wall composition of transformed callus on medium with oxalate acid | Welch *et al.* (2007) |
| *C. dentata* | Proembryogenic masses (PEMs) | AGL1, pCAMBIA 2301, *nptII*, *uidA* | Transgenic somatic embryos germinated into plants | Andrade *et al.* (2009) |
| *C. dentata* | Embryogenic tissue | AGL1, pCAMBIA 2301, *pFH1*-GUS*i*, *pFH1-GFP*, *pFH1*-GUS*i/YFP*, *pFH1-THAUM*, *nptII*, *uidA* | Acceleration of transformation using bioreactors | Kong *et al.* (2014) |
| *C. sativa* | Somatic embryo, cotyledonary node | EHA105, pUbi-GUSINT, *nptII*, *uidA*; EHA105, pK7WG2D-TAU, *CsTL1*, *nptII*, *EGFP* | Transgenic plants; transgenic plant from cryopreservation | Corredoira *et al.* (2005, 2007, 2012, 2015); see also Maynard *et al.* (2008) |
| *Eucalyptus* | | | | Chauhan *et al.* (2014) review |
| *Camaldulensis* | Leaf | EHA105, pCambia3301, *nptII*, *uidA* | Particle bombardment and *A. tumefaciens*; transient GUS expression | Mendonça *et al.* (2013) |
| *E. globulus* | Hypocotyl with or without shoot apex | EHA105, pBI121, pGW23codA, *coda*, *nptII*, *uidA* | Transgenic plants with salt tolerance | Matsunaga *et al.* (2012) |
| *E. globulus* | Shoot with leaves | AGL1, pBIN19, *EgCCR-GFP-GUS*, *nptII*, *uidA*, *gfp* | High GUS expression associated with vascular tissue | de la Torre *et al.* (2014) |
| *E. grandis* | | | | Plasencia *et al.* (2016) |

**Table 4** (continued)

| Transformant species | Explant | *Agrobacterium* strain**/plasmid/ | Results | References |
|---|---|---|---|---|
| | Radicle from 3-day-old seedling, hypocotyl-stem of 14-day-old seedling | *A. rhizogenes* A4, A4RS, ARqua1, pCAMBIA, *EgCCR1*, *gfp*, DsRed | Hairy roots, functional studies, transgenic roots with reduced lignin levels and thinner cell walls | |
| *E. polybractea* | Leaf | AGL!, pMDC204, *mgfp6*, *hpt* | Transgenic plants | Fernando *et al.* (2016) |
| *E. saligna* | Leaf | EHA105, pBI121, P5CSF129A, *nptII*, *uidA* | Transient GUS expression | da Silva *et al.* (2011, 2013) |
| *Fraxinus americana* | Hypocotyl | EHA105, pq35GR, *nptII*, *uidA*, *EGFP* | Transgenic plants | Palla and Pijut (2015) |
| *F. pennsylvanica* | Hypocotyl | EHA105, pq35GR, *nptII*, *uidA*, *EGFP* | Transgenic plants | Du and Pijut (2009) |
| *F. profunda* | Hypocotyl | EHA105, pq35GR, *nptII*, *uidA*, EGFP | Transgenic plants | Stevens and Pijut (2014) |
| *Juglans nigra* | Somatic embryo, leaf | EHA105, pMOG410, *uidA*, *nptII* | Gene expression | Fang and Wang (2000) |
| *J. nigra* | Somatic embryo | GV3101/pMP90, pBI121, pBISN1, *nptII*, *uidA* | Establishment of transgenic lines | Michler *et al.* (2008) |
| *J. regia* | Somatic embryo | EHA101, pTIBo542, *APH(3 )II*, *nptII*, GUS | Transgenic plants; GUS expression; transformation protocol | Leslie *et al.* (2006) |
| *J. regia* | Pistils | AGL1, pWBVec10a, *CBF3*, *hpt*, *uidA* | Transformation *via* pollen tube pathway | Shi *et al.* (2012) |
| *J. regia* | Somatic embryo | C58.C1,*jrPPO1* | PPO-silenced transgenic plants | Araji *et al.* (2014) |
| *J. regia* | Somatic embryo | LBA4404, C58, pBI121, *nptII*, *uidA*, p6u-ubi-FVTI, *hpt*, *fld* | Transgenic plants with osmotic stress tolerance | Sheikh Beig Goharrizi *et al.* (2016) |
| *J. regia* | Leaf | EHA105, *JrsHSP17.3* | Transient expression for temperature and salt stress tolerance | Zhai *et al.* (2016) |
| *Liquidambar formosana* | Leaf | pBin438, *Rd29A*, *nptII*; EHA105, pNOV2820, *pmi*, pCAMBIA1300, pBSNHX, *AtNHX1* | Transgene expression for salt, cold, and drought stress; transgenic plants with salt tolerance | Zhuo *et al.* (2007); Qiao *et al.* (2010) |
| *Populus alba* 'Villafranca' | Internode | EHA105, pTiBo542, C58, pBI-BAR, *bar*, *nptII* | Transgenic plants with herbicide resistance; nuclease-producing bacteria in soil | Balestrazzi *et al.* (2007) |
| *P. alba* | Leaf | LBA4404, pBE2113-GUS, *AaXEG2* | Enhancement of growth and cellulose accumulation by overexpression of xyloglucanase; biosafety assessment prior to field trial | Taniguchi *et al.* (2008) |
| *P. alba* 'Villafranca' | Internode | EHA105, pGA111, *StSy* | High accumulation of resveratrol glucosides, no increased resistance to fungal pathogen; Seasonal- and tissue-specific expression | Balestrazzi *et al.* (2011) |
| *P. alba* 'pyramidalis' | Dormant lateral bud | AGL1, pCAMBIA1305.1, GUS Plus; AGL1, pTiBO542, pCAMBIA1305.1, GUS Plus | Distinct and specific GUS-staining patterns (sectors) in secondary stem tissue; Induced somatic sector analysis | Spokevicius *et al.* (2006), Van Beveren *et al.* (2006) |
| *P. alba* 'Villafranca' | Internode | EHA105, pBI-VHb, *nptII*, *vhb* | No significant differences between VHb and wild-type plants | Zelasco *et al.* (2006) |
| *P. alba* | Leaf plastid | pCB1GFP, *accD*, *rbcL*, *aadA*, *gfp* | Biolistic bombardment, site-specific integration of foreign genes | Okumura *et al.* (2006) |
| *P. alba* 'Villafranca' | Internode | EHA105, pTiBo542, pIPT5, pIPT10, pIPT20, *ipt*, pROL20, *rolABC*, pEXM2 | Production of marker-free transgenic plants | Zelasco *et al.* (2007) |
| *P. alba* | Leaf, stem | LBA4404, pEL2Ω-DnaK, *ApDnaK* | Transgenic plants with enhanced growth rate and abiotic stress tolerance | Takabe *et al.* (2008) |
| *P. alba* 'Villafranca' | Internode | EHA105, pBI121-MT, *ipt*, *R*, *nptII*, pMAT-PsMT$_{A1}$ | Marker-free transgenic plants with tolerance to heavy metal toxicity | Balestrazzi *et al.* (2009) |
| *P. alba* 'Villafranca' | Internode | LBA4404, pMDC45, *aqua1*, gfp, *bar*, pEarlyGate-201 with HA tag | Transgenic plants with increased relative growth rate and water use efficiency under ZN excess conditions | Ariani *et al.* (2016) |
| *P. angustifolia* | Internode, axillary bud | GV3101, pPM6000, pCAMBIA-Npro-long-Luc, *luc*, *hph* | Transgenic plants | Maheshwari and Kovalchuk (2016) |
| *P. balsamifera* | Internode, axillary bud | GV3101, pPM6000, pCAMBIA-Npro-long-Luc, *luc*, *hph* | Transgenic plants | Maheshwari and Kovalchuk (2016) |
| *P. deltoides* | Leaf | LBA4404, pVST*merB*pe, *nptII* | Transgenic plants expressed high levels of MerB protein, and showed some resistance to organic mercury | Che *et al.* (2006) |
| *P. deltoides* | Leaf | C58, pCAMBIA1300, *A2pt::merB*, *merB*, *hptII*, *nptII* | Transgenic *merA* plants were re-transformed; transgenic plants expressed *merA* and | Lyyra *et al.* (2007) |

**Table 4** (continued)

| Transformant species | Explant | *Agrobacterium* strain**/plasmid/ | Results | References |
|---|---|---|---|---|
| | | | *merB*, highly resistant to phenylmercuric acetate, and detoxified organic mercury compounds more rapidly | |
| *P. deltoides* | Leaf | C5851, *A2pt::ECS* | Transgenic plants with enhanced arsenic tolerance | LeBlanc *et al.* (2011) |
| *P. deltoides* | Leaf, node, internode | LBA4404, PGA482, *nptII*, *uidA* | Transient GUS expression | John *et al.* (2014) |
| *P. tomentosa* | Leaf | EHA101, pBI121, *AhDREB1*, *nptII* | Transgenic plants with improved salt tolerance | Du *et al.* (2012) |
| *P. tomentosa* | Leaf | GV3101, pCAMBIA1301, *CBL*, *pmi* | Transgenic plants with multiple stress tolerance | Li *et al.* (2012) |
| *P. tremula* | Internode | CBE21, pBI121, pGS, *uidA*, *nptII*, *hpt* | Transgenic plants | Lebedev *et al.* (2010) |
| *Prunus serotina* | Leaf | EHA105, PsAGRNAi, *nptII* | Transgenic shoots | Liu and Pijut (2010) |
| *P. serotina* | Leaf | EHA105, pART27-PsAGRNAi, pBI121-MDL4, pBI121-PsTFL1, *nptII* | Transgenic plants, improved rooting | Wang and Pijut (2014a) |
| *P. serotina* | Leaf | EHA105, pBI121 (35S-PsTFL1), pBI121 (35S-MDL4-FLAG), pBI121 (rolC-MDL4-FLAG), pCAMBIA2301 (rolC-PH3-FLAG) | Transgenic plants overexpressing *TFL1* | Wang and Pijut (2014b) |
| *Quercus robur* | Somatic embryo | EHA105:p35SGUSINT, *nptII*, *uidA* | Transgenic plants, use of temporary immersion system (TIS) | Mallón *et al.* (2013) |
| *Q. robur* | Somatic embryo | EHA105, pK7WG2D-TAU, *CsTL1*, *EGFP*, *nptII* | Transgenic plants, use of TIS | Mallón *et al.* (2014) |
| *Q. suber* | Somatic embryo | EHA105, LBA4404, AGL1, pBINUbiGUSint, *nptII*, *uidA*; AGL1, pBINUbiGUSint or pBINUbiGUSint, pBBR1MCS-5.*virGN54D*; AGL1, pBINUbiBAR, *nptII*, *bar* | Transgenic plants; Improved genetic transformation; cryopreservation of transgenic lines with stability and expression of transgenes after 3 months | Álvarez *et al.* (2006, 2009, 2014); Álvarez and Ordás (2007) |
| *Q. suber* | Somatic embryo | C58C1pMP90, pBI121, nptII; EHA105, pUbiGUSINT or p35SGUSINT, *nptII*, *uidA* | Transgenic plants | Vidal *et al.* (2010) |
| *Robinia pseudoacacia* | Stem, leaf | GV3101, pMP90, *hpt*, *uidA* | Transgenic plants | Arrillaga *et al.* (2008) |
| *R. pseudoacacia* | Stem | *AhDREB1* | Transgenic plants | Shen *et al.* (2008) |
| *R. pseudoacacia* 'Idaho' | | GV3101, pBIN438-$P_{35S-35S}$-*FpDREB2A* | Transgenic plants with enhanced resistance to drought stress | Xiu *et al.* (2016) |
| *Ulmus americana* | Leaf | EHA105, pSE39, ESF39A, *nptII*, *uidA* | Transgenic plants; reduced Dutch elm disease symptoms | Newhouse *et al.* (2006, 2007, 2008) |

to embryogenesis in the absence of exogenous hormones (Florez *et al.* 2015), and overexpression of *Lec2* from cacao increased the frequency of regeneration of stably transformed somatic embryos (Zhang *et al.* 2014). Similar results were observed in citrus (*Citrus sinensis*) when a *Lec1-like* (*L1L*) gene was over expressed (Zhu *et al.* 2014). Maize *Bbm* and *Wuschel2* stimulated the differentiation of neighboring cells and promoted a mixture of embryogenic and organogenic tissues (Lowe *et al.* 2016), suggesting that a similar approach might also be successful in organogenic systems. The recombinase/gene induction systems should be directly applicable to trees given the many observations of recombinase activity (Fladung and Becker 2010), and the long taxonomic range over which inducible expression systems such as by abscisic acid, osmotic stress, or heat (among others) can be effective (*e.g.*, Zhang *et al.* 2010).

## Moving forward

GE innovations have emerged rapidly for trees in recent years, and there have been a very wide variety of traits demonstrated, most of which remained consistent over years in field trials. In addition, adverse (pleiotropic) effects have been uncommon or confined to specific events or environments. For stress resistance, in addition to detoxification genes that are effective in chestnut biotic stress resistance, there have been confirmed successes in citrus and plum using gene editing and/or host-induced gene silencing. The results from cold and salinity tolerance studies in the field show that even these complex abiotic stress resistance traits can be improved by GE approaches. Studies of cell walls, despite their complex underlying physiology and biochemistry, show that wood can be engineered for improved processing by altering the linkages in the main polymer backbone, and that this may not require significant yield penalties, though additional field studies are needed. Flowering modification studies have demonstrated that the onset of flowering can be advanced several years, and this capability has recently been initiated in fruit tree breeding programs. Field demonstrations of stable floral sterility, without detectable adverse effects on vegetative growth show that very high, if not complete, genetic containment is possible, potentially facilitating regulatory and public acceptance of GE plantation trees. Directed mutation through CRISPR-based gene editing systems works as efficiently and specifically for trees as it does for other crops. Transcriptomics and other genomic methods are identifying increasing numbers of genes and regulatory elements that will enable GE to be more effective and controlled, and to produce more complex traits. Transformation systems have seen some major advances very recently, and commercial applications are in place, or nearly so, in Brazil, China, and the USA. From a technical viewpoint, the table appears to be set for significant contributions of GE technologies to forestry.

However, there are also important obstacles to wider use, both biological and social. Many traits studied to date have either not been tested under field conditions similar to commercial plantations or have been in too small a sample of genotypes and environments, or for too limited a time period, to be confident that there will not be adverse effects on health or productivity.

Much of the scientific experience is based on one genus, *Populus*, because it is more amenable to GE and is grown in agronomic-style plantations. However, *Populus* may show fewer adverse effects than that would be seen in other species.

There is a need for expanded experimentation to support advanced cell wall and chemical manipulations or synthetic biology. For instance, manipulation of metabolic pathways favors major classes of cell wall components or terpenoid products without harming related physiological processes.

Transformation, including the capacity for gene editing, remains a major bottleneck for most forest tree taxa. Much of the knowledge on transformation methods resides as proprietary information within companies, where it is generally not available for broader progress. Most worrisome perhaps is that, because there appears to be declining public and private sector support for forest GE research and training programs in academia in many countries, there appears to be declining competence for advancing the field of transformation and *in vitro* regeneration studies.

There are also worrisome regulatory and market trends. Regulations throughout much of the world in essence presume that the GE method is hazardous requiring intensive and costly examination of each gene insertion event for safety and efficacy. As a presumed hazard, these events must all be fully contained during the many years to decades of breeding research and testing that is common to forestry—which from a practical point of view is extremely difficult or impossible in most cases. To maintain diversity in forests, there is a need to develop and use dozens of insertion events, not just one or two. Sterility systems can mitigate this problem, but in most cases, such a draconian measure is inappropriate or its cost to breeding or biodiversity is so high that it would create a larger problem than it solves (Strauss *et al.* 2017). The annual-crop-oriented, and highly politicized, regulatory system for GE crops is an extremely poor fit for the realities of most forestry programs and this situation has been exacerbated by international conventions (Strauss *et al.* 2009; Viswanath *et al.* 2012). Finally, the added costs imposed by regulations can nullify any advantages from genetic modification over traditional breeding (Castellanos-Hernández *et al.* 2011).

Perhaps gene editing, at least, type I mutagenesis or simple knockout or dropout mutations, might have a simpler path for regulatory approval. In the USA, the US Department of Agriculture has advised that they will not regulate plants if

they could have been developed using traditional methods when they are not considered "plant pest risks" (Waltz 2016a, b). However, complete removal of the gene editing machinery after mutagenesis of target loci is difficult in trees, as segregation of them away in progeny would be time consuming and require that elite clonal genotypes are reshuffled. Recombinases, as discussed above, might be an option when somatically induced, if the resulting small and inert footprint is not considered a regulatory trigger. Ribonucleoprotein types of technology (Svitashev *et al.* 2016), where editing machinery does not become inserted into the genome, is another option, if it can be made to work efficiently and in a variety of genotypes and give manageable levels of chimerism—very considerable challenges. If gene editing is subject to the same stringent regulations as are other types of GE trees, it too will be very difficult to use for forestry.

Market limitations in the form of forest certification now cover most of the major forestry operations in the world. However, all the major certification programs have banned all GE trees from use in the field, and in the international systems this is true even for research and regardless of whether they have obtained governmental authorization (Strauss *et al.* 2015). This makes it difficult and costly for companies to conduct research or breeding studies with GE trees and sends a strong signal that GE will not be accepted in the market and that investment in research and development is not worthwhile. The extreme costs and inertia of the current social control systems—both certification and regulation—are underlined by the inability of GE methods to be applied to help solve tree health problems (Strauss *et al.* 2015), which have reached crisis levels in numerous areas (Sugden *et al.* 2015). While there are no simple answers to the problem of public concern over GE-related technologies and the associated market and regulatory barriers, further engagement by scientists with the public would seem to be an important part of any solution. This engagement should address not just literacy about the genetic aspects of modifications but also concerns over the larger social and ecological context in which the modifications are used. Field trials and commercial demonstrations are particularly valuable engagement tools, and of course provide places for studies of trait value and ecological impact.

In summary, the technical progress in GE of forest trees, despite very hostile conditions common to all GE crops over the last two decades, has been extraordinary. Many distinct types of applications show great promise based on advanced lab, greenhouse, or field research. However, the inability to effectively apply GE and gene editing methods to a wide variety of species and genotypes, and extremely adverse regulation and market conditions, severely constrain the ability to apply these advances to commercial forestry on a significant scale. The biological limitations are largely soluble with continued research. However, a fundamental change in social conditions and public acceptance is also needed. This includes changes in regulations and market signals to create an environment conducive to accelerated research and commercial development. The growing demands on forests for products and ecological services—under an increasingly antagonistic and variable climate—justify a fundamental change of course.

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