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Biotechnology for bioenergy dedicated trees: meeting future energy demands

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Abstract: With the increase in human demands for energy, purpose-grown woody crops could be part of the global renewable energy solution, especially in geographical regions where plantation forestry is feasible and economically important. In addition, efficient utilization of woody feedstocks would engage in mitigating greenhouse gas emissions, decreasing the challenge of food and energy security, and resolving the conflict between land use for food or biofuel production. This review compiles existing knowledge on biotechnological and genomics-aided improvements of biomass performance of purpose-grown poplar, willow, eucalyptus and pine species, and their relative hybrids, for efficient and sustainable bioenergy applications. This includes advancements in tree in vitro regeneration, and stable expression or modification of selected genes encoding desirable traits, which enhanced growth and yield, wood properties, site adaptability, and biotic and abiotic stress tolerance. Genetic modifications used to alter lignin/cellulose/hemicelluloses ratio and lignin composition, towards effective lignocellulosic feedstock conversion into cellulosic ethanol, are also examined. Biotech-trees still need to pass challengeable regulatory authorities' processes, including biosafety and risk assessment analyses prior to their commercialization release. Hence, strategies developed to contain transgenes, or to mitigate potential transgene flow risks, are discussed.

Keywords: bioenergy; *Eucalyptus*; genetic modification; *Populus*; short-rotation trees.

1 Introduction

Energy is needed for human survival and society's development. However, energy requirements become more complicated as the world's population continues to expand. It is predicted that the primary energy demand will grow by over 30% by 2035 [1], leading to fossil fuel shortages, unless renewable and sustainable alternative sources are developed [2]. Bioenergy is a renewable energy derived from biological materials that can be converted by biological, mechanical or thermochemical processes into useful energy sources to produce heat, generate electricity and provide liquid biofuels (bioethanol and biodiesel) for the transport industry (Figure 1) [3–5].

Bioenergy crops not competing with food production and arable land use are being considered to provide biomass for liquid biofuel and energy production [6–9]. Wood biomass obtained from fast-growing trees with potential coppicing systems, e.g. poplar, willows, eucalyptus species, and their relevant hybrids, is a promising lignocellulosic feedstock considering its great availability. In addition, the multi-years rotations of such candidate trees allow for extended periods between successive harvests, and with limited land disturbance [10]. However, liquid fuel production from lignocellulosic feedstocks is challengeable due to biomass complexity and the presence of recalcitrant lignin [11–13]. It is more difficult than simple fermentation of sugars, or bioconversion of starch to fermentable sugars from first-generation biofuel crops, such as sugarcane and corn [4, 11]. The development of enhanced second-generation of energy-dedicated woody crops, and improvement of the efficiencies of biorefineries, will likely shift the economic balance towards more economically competitive applications of biofuels [14, 15].

The global forest area has been estimated at slightly <4 billion hectares (ha), which is 30.6% of the terrestrial land area [16]. Although most woody biomass used for bioenergy comes from natural forests, which represent 93% of the world's forests in 2015 [16], the tremendous potential for increasing bioenergy production relies on establishing short-rotation plantations. Therefore, countries that have no fossil fuel resources, but have forest plantations capability, may exploit lignocellulosic woody biomass as an alternative source for energy supply [17]. Multidisciplinary research is still required for the purpose of efficient and sustainable bioenergy and biofuel production from candidate second-generation woody crops, which are mostly undomesticated and are in the first stages of development and management [4]. Additionally, the enhancement of many bioenergy tree crops has

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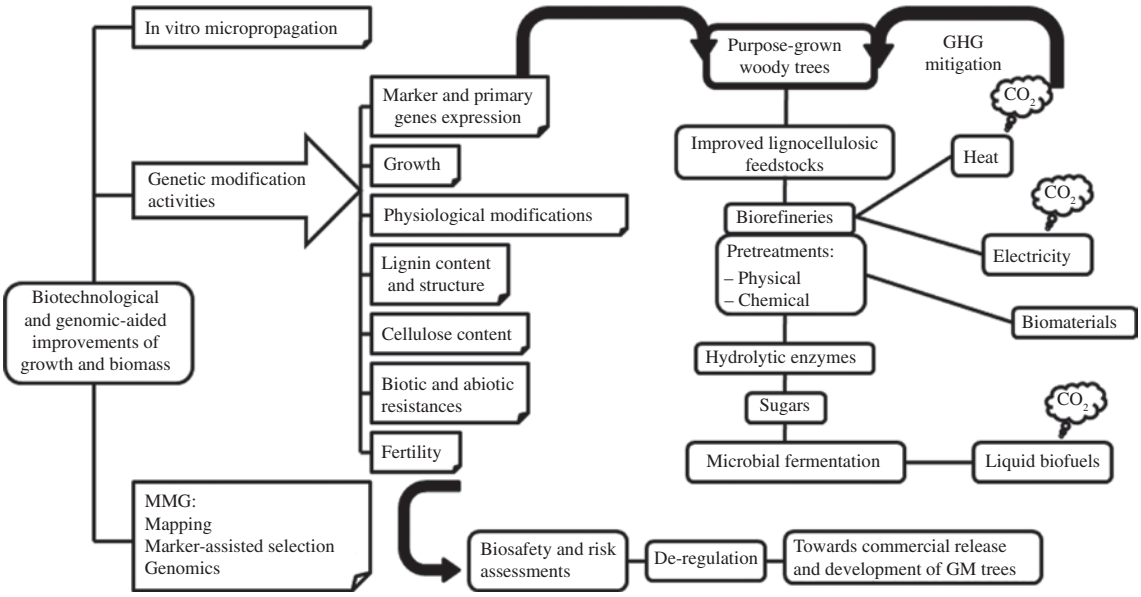

Figure 1: Overview of biotechnological and genomics-aided improvements of growth and lignocellulosic biomass in bioenergy purpose-grown trees.
Genetic modification of targeted tree traits aim to enhance wood properties and processing will pave the way towards efficient bioconversion of woody cellulosic biomass into biofuels and other energy applications. Biosafety and risk assessment analyses are needed to avoid uncertain ecological consequences of transformed genes, and to satisfy regulatory requirements and public acceptance. GHG, greenhouse gas emissions.

several breeding challenges such as long generation time, obligatory outcrossing that prevents the creation of inbred lines for classical genetic studies, and large and complex genomes [18]. Thus, a combination of advanced agricultural practices, conventional breeding and selection, and modern biotechnological and genomics aspects are necessary to accelerate forest trees domestication [19, 20], and for biomass improvement in energy-dedicated woody crops [21] (Figure 1).

2 Biotechnological enhancement of biomass energy tree crops

Forest biotechnology and genomics include modern tools and methods and their applications in agricultural and forest science, including genetic engineering, DNA genotyping, technologies for locating, identifying, comparing or otherwise manipulating genes, in addition to aspects of tree breeding and plant propagation [22, 23]. With the aid of gene transfer technology, desirable genes encoding traits of improved growth and wood properties, and tolerance to natural biotic and abiotic stresses can be introduced and expressed stably and efficiently in a reliable and reproducible propagation system [10, 24–26]. Pest resistant genes are crucial to avoid reduction in biomass productivity of target woody trees [27]. The mutual relations between growth and chemical wood properties are central, thus, genetically modified (GM) trees qualified for commercial plantations need to produce high stem biomass and have wood properties suitable for conversion into renewable biofuels [28, 29]. Woody biomass consists mainly of cellulose, hemicellulose and lignin, with various proportions among tree species and cell types within a species [30] (Table 1). The interactions between these biopolymers hinder hydrolytic enzymes accessibility, and microbial and chemical deconstruction of lignocellulosic biomass, collectively known as "biomass recalcitrance" [60]. Lignin constitutes about 20%–30% of wood composition (Table 1). It is a complex biopolymer composed of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, which are derived from three monolignols encoded by at least ten gene families in trees [61, 62]. The in planta overexpression or suppression of these monolignol biosynthesis genes using sense, antisense or RNA interference (RNAi) approaches resulted in alternation of lignin content or monolignol ratio [e.g. 12, 59]. Modified woody feedstocks with lower lignin content and higher S-to-G ratio are easier for chemical pretreatments, improve hydrolytic enzymes accessibility and cellulose digestibility [63, 64], and ultimately simplify the process of wood biomass-to-ethanol conversion [59, 65, 66]. However, lignin can probably be converted into

Table 1: Currently available worldwide information of the percent dry weight contents of major components from dedicated biomass purpose-grown trees, and potential for bioethanol production, from the peer-reviewed literature survey.

Biomass	Age at harvest (years)	Typical annual yield (dry tons acre^{-1})	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Lignin S:G	Ethanol (L kg^{-1} dry mass)	References
Poplar spp. and hybrids	3+ {1}[a] 6–7 {2}	2.35–4.8 {3} 2.83 {1} 4.5–9 {2} 7 {4}	~42–49 {5} 42.23–47.55 {6} 44 {7}	16–23 {5} 20 {7}	15.4–23.4 {8} 15.7–27.9 {9} 17.68–23.66 {6} 21–29 {5} 26 {7} 29.1 {1}	1.02–1.68 {9} 1.60 {5} 1.65–2.77 {6} 1–3 {8}	0.35 {1}	{1}[31] {2}[14] {3}[32] {4}[11] {5}[33] {6}[34] {7}[35] {8}[36] {9}[37]
Salix spp. (willow)	3–4 {2; 21}	2.8–3.9 {10} 2.83–9.71 {11} 3.2–7.3 {12; 13} 3.8–12+ {2} 4.7 {14} 5.7–8.9 {15} 10.93 {16}	34.8–41.8 {17} 35–45 {17; 18} 39–45 {18}	30.1–33.6 {19} 31–35 {17; 18}	20–23 {18} 20–29 {17; 18} 23.9–28.8 {17} 25.2 {1}	–	~0.31 {20} 0.32 {1}	{10}[38] {11}[39] {12}[40] {13}[41] {14}[42] {15}[43] {16}[44] {17}[45] {18}[46] {19}[47] {20}[48] {21}[49]
Eucalyptus spp. and hybrids	3+ {1} 3–7 {2} 6–8 {22}	2.83–6.1 {1} 3.6–7.3 {22} 4.86–8.90 {23} 4–14.3 {2} 9 {4} 10–14.6 {24}	48.07 {5}	12.69 {5}	25.9–33.2 {25} 26.91 {5}	1.72 {5}	~0.35 {26}	{22}[50] {23}[51] {24}[52] {25}[53] {26}[54]
Pine spp.	8–10 {2} 15+ {25; 27; 28}	3.3–8.5 {2} 4 {29} 5.1 {4} 9.3 {24}	~42 {1}	~27 {1}	26.8 {30} ~28 {1}	NA	0.273 {31}	{27}[10] {28}[55] {29}[56] {30}[57] {31}[58]

[a]Serial numbers between curly brackets {} are corresponding to the appropriate references.

NA, Not applicable; as conifers softwood is characterized by a guaiacyl-rich (G-type) lignin, and the absence of syringyl (S-type) subunits [59].

synthesis gas via gasification or applied directly as solid fuel [reviewed in 67]. In addition, the former modifications have produced lignin with future targeted properties for recovery and downstream conversion into value-added products. Coupled with genetic engineering; advances in analytical chemistry, computational modeling, and the advent of biorefineries and pretreatment technologies will enable new uses for this biopolymer, including high performance plastics and thermoplastic elastomers, polymeric foams, and a variety of commodity chemicals [68, reviewed in 69].

In parallel to genetic engineering technology, genomics has become a key tool for the analysis of plants and their performance, and would provide information on the identity, location, impact and function of target genes [70]. Applications of genomics to plant-based energy projects have recently been invested. This allows for the identification of an array of target genes for maximizing biofuel yield, screening and selecting superior energy genotypes, and thus accelerating the domestication and improvement of candidate plants for bioenergy and biofuel applications [70–72]. In addition, the ongoing determination of genome sizes and sequences of forest trees, provides a platform for subsequent analysis of their genetic potential, accelerating the rate of breeding, and helps selection of desired genes for the improvement of candidate woody varieties [23, 73]. Moreover, molecular markers are valuable tools in assessing genetic diversity in germplasm collections, to map important quantitative traits and to select for desirable traits linked to those markers [e.g. 23, 74].

Although many forest species may have good potential for bioenergy and biofuel applications, the greatest emphasis on tree biomass has been placed on poplar (*Populus* spp. and relative hybrids), willows (*Salix* spp.), *Eucalyptus* and temperate pines (*Pinus* spp.) [15]. Thus, the main objective of this review is to address the progress made in biotechnology and genomics, towards crop-improvement of such candidate purpose-grown and fast-growing woody species, targeted for cellulosic ethanol and bioenergy production, with emphasis on genetic modifications suited to maximize their bioenergy potential.

2.1 Poplar

Poplar is a fast growing species belonging to the genus *Populus* in the family *Salicaceae*. There are about 30 natural species of *Populus*, mostly deciduous trees that are distributed widely throughout the Northern Hemisphere. Hybrids within and among poplar species belonging to two sections, *Aigeiros* and *Tacamahaca* (cottonwoods), are commonly referred to as "hybrid poplars" [14]. Poplars and their relative hybrids comprise a woody biomass option for many temperate and cooler climates [17]. It is estimated that for every unit of energy needed to manage poplar short-rotation system, 14 units of energy are produced [75]. However, compared to other trees, poplar wood is less suitable as primary energy source because of its low density (0.56 g cm^{-3} [76]), and high water content at harvest time (55%–60% [77]), being mainly considered as a potential alternative for cellulosic ethanol production [78]. Fast-growing hybrid poplars have about 6–7-year rotations [14, 77], and annual biomass accumulation between 2.4 and 9 dry tons per acre per year depending on the genotype, plantation system, climatic and nutritional factors [14] (Table 1). These data are primarily from research plots, as yields from commercial plantations are proprietary and not readily available. In addition to conventional breeding and selection approaches, improvements in poplar wood quality and yield are amenable with the aid of biotechnological and emerging "omics" approaches, and marker-assisted breeding studies of genes and alleles that influence growth, tolerance to variable stresses, cell wall structure, and control lignocellulose biosynthesis [64, 79–81]. Furthermore, genetic improvements of poplar using genes influencing metabolism of nitrogen, branching, stem thickness, light response competition and plant height were also examined [59, 82].

Poplar has been established as a model tree for woody perennial plants in many aspects of biology and genomics studies [79, 83, 84]. It has a small genome size among tree species [=422.9 Mbp, Phytozome v3.0, Joint Genome Institute, US Department of Energy (JGI, DOE): http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Ptrichocarpa]. The annotated genomic sequence of *P. trichocarpa* is a complement to the 125,000 expressed sequence tags from poplar that has been deposited in public databases [85, 86]. As part of the poplar genome sequencing project, a full length collection of 4664 complementary DNA clones have been generated, which are useful for gene prediction associated with defense against insects [87].

Poplar and several of its relative hybrids are feasible to genetic engineering approaches [88, 89]. Overexpression of *P. trichocarpa* salt overly sensitive 2 genes (*PtSOS2*) [90], and of the chickpea *CarNAC3* gene [91], enhanced salt tolerance in genetically modified (GM) hybrid poplar plants compared to the non-transgenic controls. The ectopic expression of the *Arabidopsis* C-repeat binding factor transcriptional activator (*CBF1*) in *Populus* increased their freezing tolerance [92]. Field trials of GM poplar are being evaluated for altered wood composition and properties, sterility, lignin modification, herbicide tolerance, insect tolerance, faster growth and phytoremediation [25, 93]. China, globally ranked sixth in biotech crop hectarage [94], has been granted a commercial-scale cultivation of GM poplar trees engineered with insect resistance genes including the *Bacillus thuringiensis* (*Bt*) gene. The *Bt* gene appears to be an effective tool for protecting against leaf beetle damage and improving growth in field-grown hybrid poplar clones [93].

Research so far has shown that overexpression of the cytosolic glutamine synthetase gene (*GS1a*) significantly promotes vegetative growth, increased tree height, enhanced efficiency of nitrogen assimilation and tolerance to water stress, and enhanced resistance to phosphinothricin herbicide in the engineered poplar clones [e.g. 95–97]. Field grown hybrid poplar clones transformed with the same transgene exhibited altered fiber and wood chemistry with significant increase in wood fiber length, wood density and microfiber angle [98]. Additionally, these glutamine synthetase (GS) engineered trees showed elevated concentrations of wood sugars, specifically glucose, galactose, mannose and xylose, and significant reduction in total extractive content and acid-insoluble lignin when compared with wild-type poplar trees. These characteristics resulted in improved lignin solubilization with no concurrent decrease in yield [98]. The overexpression of *Aspergillus* xyloglucanase in *P. alba* enhanced internode length and stem growth, and increased cellulose content in transformed poplar grown in a growth chamber [99]. Nevertheless, it is always important to implement field

trials of appropriate duration, especially when transgenics are involved. In this respect, the GM poplar that overproduced xyloglucanase grew 40% taller than the wild type in growth chambers, but subsequently grew poorly in field trials [100], and showed morphological abnormalities and physiological dysfunctions [101]. Moreover, expression of the *Arabidopsis* endo-(1-4)-β-glucanase gene (*cel1*) in poplar trees resulted in longer internodes than those in wild-type controls [102]. The overexpression of the *Arabidopsis* nucleoside diphosphate kinase 2 (*AtNDPK2*) antioxidant gene driven by an oxidative stress-inducible SWPA2 promoter, enhanced growth and tolerance of GM poplar plants to oxidative stress. These transgenic poplar trees showed increased branch number and stem diameter when grown under field conditions [82]. Increasingly, Gray-Mitsumune et al. [103] reported accelerated growth in GM hybrid poplar trees that expressed expansin gene (*PttEXPA1*), which caused stem internode elongation and leaf expansion by enhancing cell wall extension. In a recent study, wood reinforcement of a poplar hybrid was demonstrated by Sakamoto et al. [104], using a rice secondary-wall thickening transcription factor (NST). The transgenics exhibited densified fiber cell walls, an increase in stem cell wall content by 39%, and a 57% increase in the stem physical strength. Alternatively, the tree "green-revolution" using semidwarfism genes could be of value for biofuel purposes. Researchers at the Oregon State University reported the development of shorter GM poplar hybrids (*P. tremula*×*P. alba*) transformed with the *GA Insensitive* (*GAI*) and *GA 2-oxidase* genes that affect in planta gibberellins (GA) action [105]. These trees exhibited advantageous alterations in several traits of growth rate, biomass production, branching, water-use efficiency and root structure. The researchers also found that the semidwarf poplar trees had dramatically reduced growth when in direct competition with tall wild-type trees, which could be effective tools to mitigate the spread of exotic, hybrid, and GM plants in wild and feral populations, thus, reducing potential risks of transgene flow.

The above-ground biomass of poplar species and hybrids comprises about 44% cellulose, 20% hemicellulose and 26% lignin [33–35] (Table 1). Successful efforts of manipulating key enzymes involve in the lignin metabolic pathway produced poplar clones with diverse transgenic wood properties such as low lignin, a high S/G ratio, a high cellulose/lignin ratio, and various combinations of these traits [e.g. 59, 64]. The extent of lignin content reduction and monomeric composition alteration, and the growth performance of GM trees, depend on the gene manipulated, the approach used, and the culturing conditions practiced. A negative correlation between biomass growth and lignin content of wood was observed in GM poplar lines by constitutive suppression of the *Pt4CL* gene encoding 4-hydroxycinnamate CoA ligase in the monolignol biosynthetic pathway [106]. Downregulation of the *4CL* caused a 45% reduction of lignin that was compensated for by a 15% increase in cellulose and 17%–57% increase in hemicelluloses. This cell wall alteration enhanced root, leaf and stem growth without affecting the structural integrity of the GM poplar plants [106]. Similar lignin/cellulose ratio was reported by Li et al. [107]. However, the above pleiotropic growth enhancements were not noted in GM poplars having *Pt4CL* downregulated with a xylem-specific promoter, which might account for the normal growth phenotypes of these transgenic clones [107]. In contrast, Voelker et al. [108] found that the *4CL* downregulation in field-grown poplar hybrid transformants did not correlate with increased growth rates or saccharification potential, in addition to important physiological vulnerabilities when lignin contents were strongly reduced. Similar transformants with antisense *4CL*, showed 30% increase in total lignin content by the second coppice [109]. Thus, it is rational to focus on promising construct lines, and to combine greenhouse studies with further extensive field trials that go a long way to obtaining physiologically meaningful results and be a guide for efficient and ecologically sound development of GM poplar varieties for efficient and sustainable biofuel applications. Similar to *4CL*, manipulating other key genes involved in lignin monomer biosynthesis caused alterations in lignin content and composition. Downregulation of the cinnamoyl-CoA reductase (CCR) reduces lignin content in the engineered poplar events by up to 50%, with an increased proportion of cellulose and ethanol yield [66, 110]. A 17% reduction in lignin were also achieved in caffeic acid O-methyltransferase (COMT) downregulated transformed poplars with complete lack of syringyl units, confirming that COMT essentially contributes to the formation of lignin S monomers [111]. A similar role was ascribed to the *F5H* gene; a cytochrome P450-dependent monooxygenase [112]. The over-expression of the *AtF5H* gene under the cinnamate 4-hydroxylase (C4H) promoter caused a significant increase in lignin S units and an increase in pulping efficiency, compared to non-transgenic control [113–115]. The resultant transgenic lignins were more linear and displayed a lower degree of polymerization [115], which will facilitate biomass deconstruction and influence ethanol yield. In the same regard, downregulation of the *C3′H* gene encoding coumaroyl 3′-hydroxylase resulted in a decrease in total lignin content, and enhanced generation of H-units at the expense of G-units, but without affecting the S-proportion content [116].

The potential impact of the S/G lignin ratio or lignin composition on sugar release from poplar lignocellulosic feedstocks was further investigated [36, 117, 118]. Studer et al. [36] showed that natural *Populus* plants with S/G ratios less than 2.0, exhibited negative correlations between sugar release, specifically glucose release, and the lignin content. In a recent study, an RNAi-mediated downregulation of a cell wall biosynthesis *PdKOR2* gene caused an alteration in the cellulose biosynthesis pathway, and ~50% decrease in the S/G lignin ratio in xylem fiber cell walls of transgenic *P. deltoides* events comparing to non-transgenic control [119]. This significant change in the S/G lignin ratio is believed to be a contributor to the stunted poplar growth properties observed in a previously studied *PdKOR*-RNAi transgenic poplar events, which in addition, showed reduce cellulose content and an increase in cellulose crystallinity [120]. Although few plant laccases have been functionally evaluated, it is thought that they may be involved in lignin biosynthesis, since they are capable to oxidize monolignols, which leads to higher order lignin formation. Recently, it was shown that downregulation of a laccase gene in *P. deltoides* (*PdLAC2*) conferred increased S/G ratio and sugar release, and increased the above-ground biomass compared to controls [118]. These phenotypes were consistent with reduced recalcitrance of plant biomass to bioconversion, which is interestingly, dependent on a mild pretreatment prior to chemical extraction of sugars. Similar biomass-related phenotypes were also reported by Biswal et al. [117] in transgenic *P. deltoides* expressing an antisense construct of the *GAUT12*, a gene encoding glycosyltransferase, which is proposed to be involved in secondary cell wall glucuronoxylan and/or pectin biosynthesis. Therefore, a growing knowledge of the genetic regulation of plant cell wall remodeling and the impact of the resultant modified cell walls on plant growth and performance are important in guiding us to a better redesigning of sustainable biofuel trees. It may also be noted that manipulation of the lignin synthetic pathway may influence defense systems, and should, therefore, be tightly linked with new strategies to improve poplar tolerance to major pathogens and herbivores [64, 121].

Other approaches have dealt with designing poplar trees with improved lignin digestibility, thus, reducing the efforts and costs of biomass conversion to liquid fuels. This is based on incorporating monolignol ferulates conjugates into natural lignin polymers, which was achieved through engineering exotic transferase into commercially relevant poplars [122, 123]. The initial work was demonstrated by Wilkerson et al. [124] who engineered poplars with a transgene encoding feruloyl-CoA monolignol transferase from *Angelica sinensis* (*As*FMT) to produce lignin with improved saccharification potential compared to wild-type poplars. The *AsFMT* transgene is capable of forming monolignol ferulate conjugates with cleavable ester bonds, which were incorporated into the natural lignin polymer backbone, permitting easier chemical degradation. Recently, the expression of monolignol 4-O-methyltransferase (MOMT4) that chemically modifies the phenolic moiety of lignin monomeric precursors, thus preventing their incorporation into the lignin polymer, substantially altered lignin content and structure in hybrid aspens [125]. The *MOMT4* transgenics performed a 62% increase in the release of simple sugars and up to a 49% increase in the yield of ethanol when their woody biomass was subjected to enzymatic digestion and yeast-mediated fermentation [125].

2.2 Willows (*Salix* spp.)

Willows (*Salix* spp.) include about 450 species of a very diverse group of catkin-bearing trees, shrubs and prostrates, which are mostly found in the temperate and arctic zones of the Northern Hemisphere [126, 127]. The willow tree is a closely related species to the poplar in the family *Salicaceae*. Both are close in terms of growth, harvesting and biomass yield and composition (Table 1). Willows used in woody crop systems are primarily drawn from the subgenus *Caprisalix* (Vetrix), which are fast-growing, tolerant to high planting density, with high biomass yield and good coppicing ability [41, 43, 127]. The plantation stands can be maintained in 3–4-year rotations, with 7–10 harvests per plantation system before replanting [49]. The typical annual biomass yield ranges between 2.8 and 12 dry tons per acre depending on the plantation region [41, 43] (Table 1). Willows have a broad genetic diversity, and it is feasible to shorten breeding cycle systems as has been utilized in Europe and North America, to produce improved hybrid varieties with enhanced woody biomass traits and resistance to pests, diseases and environmental stresses, even with little or no knowledge of the genetic basis of these genetic traits [41, 42, 128, 129]. It was found that novel hybrids have high phenotypic variation for biomass composition, owing to differences in cell wall characteristics, with superior performance of the triploid hybrids [130–132].

The sustainability of willow crops as a renewable source for large-scale production of cellulosic bioethanol requires a whole lifecycle of environmental and economic analysis. Analysis of a willow-based bioethanol process showed that bioethanol from willow would produce over

80% less greenhouse gas than burning gasoline from fossil fuels, but with uncertain economic viability [133]. The life-cycle impact of bioethanol production from short rotation willow feedstock was further assessed by Budsberg et al. [48]. Based on the bioconversion process they used in their analysis, about 0.31 L of ethanol was produced per oven dry kg of feedstock, and the process was virtually carbon neutral.

The improvements of structural and chemical characteristics of willow wood biomass and its suitability for hydrolysis and subsequent fermentation into cellulosic ethanol are crucial. Recent studies have examined different willow genotypes with cellulose content ranged from 35% to 45%, 30% to 35% for hemicelluloses, and lignin content ranged from 20% to 29% [45, 46] (Table 1). Lignin content could be reduced by integrated systems of selective breeding and genetic engineering to contents as low as 10% in a similar manner reached in *Populus* [29]. This would significantly influence biomass pretreatment and enzymatic saccharification efficiency [11], and reduce input energy and environmental impacts of the lignocellulosic bioethanol production process [134]. On the other hand, relationships between cellulose content, sugar release, and ethanol yield, as well as wood density were identified for diverse shrub willow genotypes [46]. The response of wood biomass to pretreatments was found to be different among willow genotypes. The *S. dasyclados* clone, SV1, was the best biomass producer, and the least recalcitrant to sugar release after selective biomass chemical pretreatments suited for cellulosic ethanol production [46].

Genetic and genomic approaches in willow have benefited from advances in the closely-related poplar species, which substantially improved our understanding of the basis of biomass traits in willow for more targeted breeding via marker-assisted selection. Willow trees have a small genome size of about 379 Mb (Phytozome v1.0, JGI, DOE: http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Spurpurea_er). Altogether, the simplicity of the willow's genome, current efforts towards genome-sequencing and efficient utilization of significant 'omics' and mapping technologies [e.g. 74, 129, 135–137] will facilitate the development of novel molecular breeding and selection strategies for candidate traits associated with growth, wood performance and resistance to biotic and abiotic stresses. Genetic-based strategies to dissect complex traits into more defined components for molecular breeding and gene discovery in different willows genotypes are ably discussed in a review by Hanley and Karp [129].

Willow trees are easy to propagate vegetatively using hardwood cuttings and seeds. However, many *Salix* species are considered to be recalcitrant to efficient in vitro regeneration and genetic transformation, which might be an obstacle towards development of GM willows for useful biotechnological applications. Grönroos et al. [138] induced indirect somatic embryos from callus derived from floral explants of *S. viminalis*, but growth into plants rarely occurred. Vahala et al. [139] were the first to transform *Salix* stem segments and produce transgenic callus. However, Vahala et al. [140] used the same method to express a cytokinin synthesis gene from *A. tumefaciens* T-DNA in *S. viminalis*, but the transformed callus was non-morphogenic. Further characterization of leaf calli and limited in vitro shoot micropropagation from inflorescences, shoot apices, and nodal segments of *Salix* species were also reported [141, 142]. Recently, Yang et al. [143] reported an *A. tumefaciens* transformation method for *S. matsudana*. In this study, multiple shoots were induced directly from embryonic shoot apices, and transgenically-stable plants engineered with β-glucuronidase (GUS) as a reporter gene were successfully developed. In conclusion, further development and optimization of sufficiently efficient in vitro regeneration and genetic transformation techniques for desired *Salix* species and cultivars are required to fulfill prospective large-scale genetic improvements of willow species as candidate crops in bioenergy production system.

2.3 Eucalyptus

Eucalyptus, which is native to Australia and its northern neighbors, belongs to the family *Myrtaceae*, and encloses a large genus of trees and large shrubs of more than 700 species and hybrids. Less than 15 species of eucalyptus are commercially significant worldwide. Primarily, eucalypts are the most planted hardwood trees in tropical and subtropical parts of the world [144]. *Eucalyptus* spp. has been domesticated for various products with 20 million ha of plantations in about 90 countries [145]. *Eucalyptus grandis* is the most widely cultivated species in subtropical and warm temperate regions [146], *E. camaldulensis* is a common species of arid and semi-arid regions, while *E. globulus* grows predominantly in temperate climates free of severe frosts. The species *E. urophylla* is highly productive, whereas *E. nitens* and *E. amplifolia* are important cold adaptable species [14, 146, 147].

Many species of *Eucalyptus* and their relevant hybrids have high growth rate, high biomass density, self-pruning, ability to coppice, and tolerance to environmental stress, making them good candidates for the production of high-quality wood biomass for timber and firewood, paper

making, and for extraction of high-value chemicals [53, 148, 149]. In addition, improved *Eucalyptus* hybrids offer a promising renewable lignocellulosic feedstock for bioenergy production [50, 51, 54, 150]. In general, eucalyptus biomass yields are influenced by precipitation, fertility, soil type and soil nutrition, location, management, and genetics [14, 151] (Table 1). Comparable studies on eucalyptus potential yields between 4 and 14.3 dry tons per acre per year were reported in US-DOE [14], where subsequent analysis assumes a conservative annual yield average of 6.0 dry tons per acre in southern US plantations. Even though, conventional breeding programs of eucalyptus have some limitations due to long breeding cycles, high levels of heterozygosity and incompatibility barriers [151]. Such programs are mostly dependent on repetitive selection and/or inter-specific hybridization [147, 151]. Eucalyptus is commercially propagated by both seed and stem cuttings [151]. Although in vitro micropropagation protocols are available [152], many *Eucalyptus* species and elite genotypes are still considered recalcitrant to tissue culture, and thus one of the main limitations restraining the wider application of genetic engineering strategies to this genus. In general, genetic transformation of eucalyptus is in its infancy, and commercial utilization of GM eucalyptus is at its initial stage due to obstacles such as the problem of loss of regeneration and rooting ability, limited success in generating GM plants, poor information on transgene flow, and most data are kept confidential by private companies and protected by patents [147, 153]. Nevertheless, the regenerable and transformable species including *E. grandis* and *E. camaldulensis*, are being exploited as models within the genus for breeding, gene transfer and advanced genomics analyses [e.g. 153–155]. In this respect, the recent release of *E. grandis* genome sequence, in addition to advanced genetic maps and cytogenomic studies dealing with karyotyping comparisons between economically important *Eucalyptus* species, will increase our understanding of the *Eucalyptus* genome structure and organization and will orient successful advanced breeding strategies [144, 156–158]. Furthermore, the former advancements will provide a valuable resource of candidate genes, which will accelerate the development of transgenic eucalypts with novel traits dedicated to a wide range of uses including biofuel production. Advances in lignocellulosic biofuel production from eucalypt biomass are ably reviewed in [150].

Several studies and improvement programs focused on genetic engineering of Eucalyptus spp. and their elite hybrids indicated that eucalyptus is a promising biomass for bioenergy production [10, 24, 25, 153]. Biomass enhancements included wood quality through improvement of cellulose biosynthesis or modification, biomass increase and lignin modification. For example, overexpression of the *cbd* gene (encodes for cellulose binding domain that modulates the elongation of plant cells), and the *cel1* gene (coding for endo-1,4-β-glucanase that involved in cell wall enlargement) in *E. camaldulensis*, and in *E. grandis* and its hybrids, resulted in faster growth of the regenerated GM plants [159, 160]. In addition, transgenic tobacco plants overexpressed the *E. camaldulensis* transcription factor (*EcHB1*) gene related to xylem development; showed greater fiber length (20%) and increased plant height (50%) when compared to the wild type [161]. In Brazil, ~3.5 million ha of fast-growing hybrid eucalyptus trees are grown around the country. The GM eucalyptus trees have been engineered with an *A. thaliana* gene encodes a protein that accelerated plant growth by facilitating cell-wall expansion [162]. Accordingly, fast-growing biotech eucalyptus trees produce 20% more wood than conventional trees and are ready for harvest in 5.5 years instead of 7. These findings would be reasonable for biomass enhancement in Eucalyptus species as purpose-fast growing trees, especially if get reinforced with improved tolerance to biotic and abiotic stresses. In this respect, insect resistant and herbicide tolerant *E. camaldulensis* was developed by Harcourt et al. [163] via *A. tumefaciens*-mediated genetic transformation, using the insecticidal *cry3A* gene and the ammonium glufosinate resistant *bar* gene. New insights into an RNAi approach for the control of a serious pest in eucalyptus plantations was reported by Nambiar-Veetil et al. [164]. Shao et al. [165] introduced the *cecropin D* gene into *E. urophylla*, and the transgenic plants showed increased resistance against the wilt disease caused by the bacterial pathogen, *Pseudomonas solanacearum*. Additionally, over-expression of choline oxidase (*cod*A) transgene from *Arthrobacter globiformis* in *E. camaldulensis* and *E. globules*, induced tolerance of the GM plants to salinity and extreme temperatures [166, 167].

Other studies of importance included the in vitro regeneration and genetic transformation of a highly productive tropical *Eucalyptus* hybrid (*E. grandis* × *E. urophylla*). A mitochondrial citrate synthetase gene (*mtCS*) engineered into this hybrid genome; enhanced phosphorus uptake from acidic soils five fold compared to non-transgenic controls [168]. An *Arabidopsis* transcription factor *CBF2* that up-regulates the cold-response pathways in plants has also been engineered into the genome of the (*E. grandis* × *E. urophylla*) hybrid (reported in: [10]). This new freeze-tolerant variety has demonstrated tolerance to ~ −9 °C across multiple years and multiple field trial locations, while essentially maintaining its exceptional productivity. This will

allow efficient growing of such freeze-tolerant *Eucalyptus* varieties in sites other than the tropics. For example, in the USA, cold tolerant eucalyptus is currently growing in pilot scale trials in the southeast USA [148]. Developing freeze-tolerant commercial varieties would expand the potential plantation range from Southern Florida into cooler regions as far as South Carolina, and enable the selected trees to survive the winter [14]. In another investigation, Navarro et al. [169] over-expressed two transcription factors genes (*EguCBF1a/b*) from *E. gunnii* in a cold-sensitive *Eucalyptus* hybrid. In addition to improved freeze tolerance, some CBF-transgenic events showed pleiotropic effects such as higher levels of anthocyanins, fewer stomata, wax deposition on cuticle, reduced leaf area, and better water retention capacity under cold stress when compared to non-transgenic control.

To improve enzymatic saccharification of eucalypt biomass, research attempts were made to decrease lignin content in wood, or alter its structure. Chen et al. [170] introduced sense and antisense constructs of an aspen *C4H* gene into *E. camaldulensis*. This transgene coded for lower lignin content in both orientations and resulted with superior biomass of transgenic shoots compared to the non-transgenic controls. More recently, in 2011, ArborGen Inc., and their collaborators developed a downregulated C4H (*E. grandis* × *E. urophylla*) hybrid line. The transgenics had only half of the lignin content, grew well, and yielded twice the sugar compared to non-transgenic controls. These C4H-GM trees were estimated to produce roughly 10 dry tons per acre per year of biomass, and nearly 1000 gallons of biofuels per acre (http://biomassmagazine.com/articles/7038/genetically-modified-low-lignin-eucalyptus-yields-twice-the-sugar/?ref=brm). Similar C4H-GM eucalypt hybrids were further generated via RNAi-down-regulation of lignin biosynthetic genes *C3H* and *C4H* [171]. The total lignin content in both transgenic lines was reduced by 8%–9%, and the sugar release was increased (94% saccharification in *C3H*, and 97% in *C4H*) compared to control biomass (80%). However, the transgenic lines were dwarfed, highlighting the influence of the genetic modification approach being used on growth and performance of the developed GM events. Other approaches used antisense sequence of the *cad* gene to downregulate cinnamyl alcohol dehydrogenase (CAD); the last enzyme of the monolignol biosynthetic pathway [172]. Significant inhibition of CAD activity was achieved in 58% of the transgenic (*E. grandis* × *E. urophylla*) hybrid events, of which, two promising lines grown under glasshouse conditions showed 26% and 22% residual CAD activity [172]. The same antisense construct was transformed into *E. camaldulensis* by Valerio et al. [173]. Among the 44 regenerated lines, 32% exhibited up to 83% reduction in CAD activity. However, it had been reported that after 10 months of growth in a glasshouse, none of the five transgenic lines tested showed any change in lignin profiles or CAD activity when compared to untransformed control plants (quantity, composition and pulp yield). Other efforts towards lignin modifications in eucalyptus included the suppression of Ecliml, which is one of the key transcription factors involved in lignin biosynthesis in eucalyptus. Ecliml is orthologous to the tobacco LIM domain transcription factor (Ntliml) that was introduced in an antisense fashion into *E. camaldulensis* [174]. Suppression of the LIM gene caused a simultaneous reduction in the levels of transcripts of some lignin pathway genes including phenylalanine ammonialyase (PAL), C4H, and 4CL, and a 29% reduction of lignin content in the GM plants grown in the greenhouse [174]. The recent availability of the *E. grandis* genome sequence facilitates the in silico identification of 38 *E. grandis* phenylpropanoid genes involved in monolignol biosynthesis [154]. Seventeen of the 38 genes exhibited strong, preferential expression in highly lignified tissues, probably representing the *E. grandis* core lignification toolbox. This provides the foundation for the development of biotechnology approaches to develop tree varieties with enhanced biomass processing qualities.

Lastly, as eucalyptus is native to Australia, Indonesia and Papua New Guinea, this makes its plantations in tropical and subtropical countries such as Brazil and southeast USA satisfactory to regulators due to the absence of native relatives to eucalyptus, and also due to it being a non-invasive plant in most areas of these countries [162]. Recently, and for the first time globally, a GM yield-enhanced eucalyptus event developed by FuturaGene™ was approved for commercial use in Brazil after extensive biosafety assessments (http://www.futuragene.com/FuturaGene-eucalyptus-approved-for-commercial-use.pdf). Thus, it is expected that commercial plantations of eucalyptus will continue and will play a potential role as feedstocks for pulp, paper and timber, as well as for bioenergy systems.

2.4 Pine (*Pinus* spp.)

The *Pinus* genus has over 100 species, with high genetic and phenotypic diversity among pines species [175]. Loblolly (*Pinus taeda*) and slash (*P. elliottii*) are the most important pine trees in the US due to their broad natural ranges [14, 176]. Radiata pine (*P. radiata*) is extensively planted in Chile, New Zealand and Australia [177], and the long-rotation conifers such as Scots pine (*P. sylvestris*) are

widely planted in the temperate regions of Asia, Europe and North America [178], while maritime pine (*P. pinaster*) is grown in Europe [177]. Loblolly pine is the model pine because of its economic importance and its well-characterized reproduction and genetics [179]. Its wood is currently best suited for bioenergy applications that utilize direct firing or gasification technologies, although it is believed that enzymatic processes leading to cellulosic ethanol production could be utilized [57, 180]. With a capacity of 75% conversion of loblolly pine wood carbohydrates to fermentable sugars, it is concluded that ethanol produced from pines might be competitive with ethanol produced from corn or other lignocellulosic feedstocks [57, 68].

In addition to natural stands, many pine species are established in commercial plantations and could contribute to the bioenergy sector [14, 176]. In general, pines have relatively long rotation time (about 15 years for pulp wood applications and 23 years for sawtimber applications) [10], which has bioenergy ramifications. A promising annual yield of 9.3 dry tons per acre of a closely-spaced slash pine plantations was reported by Stricker et al. [52]. In parallel, loblolly pine grown to a 20-year rotation can produce an average 4 dry tons per acre per year [56], which is less than half of the productivity rate of 8–10 dry tons per acre per year as projected by the US-DOE and others [181]. Very intensive management of selected loblolly pine genotypes in southern US plantations has increased biomass yield to 5.4–8.5 dry tons per acre per year [14] (Table 1). Pines can be clonally propagated by conventional rooted cuttings, in addition to in vitro organogenesis and somatic embryogenesis [178, 182]. Commercial germplasms of cloned and selected loblolly pine varieties, which focused on growth, stem form and disease resistance, have been developed through genetics and breeding efforts. In the first two breeding cycles of loblolly pine, gains of 30%–40% in stem volume per cycle were achieved [183]. In addition, efforts are being suggested to address pine bioenergy limitations, including planting pines with relatively dense spacings and short rotations of 8–10 years, with an approximate annual harvest of 5.5 dry tons per acre under appropriate management [14]. Early research results accomplished by the ArborGen Inc. company indicated that rotation times of 15 years might be possible. This company has introduced proprietary candidate genes associated with improved growth into loblolly pine that demonstrated nearly double the normal biomass production in the first 3 years of field trials relative to control trees (reported in [55]).

Pines have massive and complex genomes, and the largest genome assembled to date, a draft genome sequence of about 20.15 Gbp of *P. taeda* has been published recently [184, 185]. It is about 48 times bigger than the genome of poplar. Altogether, significant genomics and gene discovery approaches, transcriptomics and proteomics are generating a growing body of substantial information valuable for advanced breeding programs, and genetic improvement of pines growth and wood properties, to customize pine trees for bioenergy initiatives [e.g. 23, 186, 187]. For instance, the pine genome assembly by Neale et al. [184] revealed the presence of putative homologs for many genes that encode transcription factors that regulate wood cell types or the perennial growth habit. In general, genetic transformation of conifer species is difficult, though successful transformations of diverse pine species have been achieved via biolistic bombardments and *A. tumefaciens*-mediated methods [e.g. 177, 182, 188]. This has paved the way to develop cost-efficient opportunities for deployment of genetically improved pines planting stocks. As is common to most commercial GM crops, insect-resistant strategies were also applied to pines trees. Overexpression of the synthetic *crylAc* gene of *Bt* bacteria caused effective insect resistance against *Dendrolimus punctatus* Walker and *Crypyothelea formosicola* Staud in transformed loblolly pine clones [189]. Transgenic *P. radiata* plants overexpressed the same gene and also displayed variable levels of resistance to insect damage [190]. On the other hand, advances in omics-assisted studies smoothed the way towards better understanding of coordinated control of growth regulation during stress and acclimation to long-term stress [191]. Microarray-based transcriptomics studies in pines related to drought and cold stresses are summarized in a review by Harfouche et al. [192]. Transgenic pine clones tolerant to environmental stresses were also regenerated. For example, the overexpression of the pepper *capsicum annuum* pathogen and freezing tolerance-related protein 1 (*CaPF1*) gene encoding the ERF/AP2 trascription factor, resulted in a dramatic increase in tolerance to drought, freezing, and salt stress in Eastern white pine (*P. strobus*) transclones [193]. Such transcription factor may be used to engineer susceptible bioenergy-dedicated pine species for multiple stress tolerance.

Biomass yield and quality are primary targets for genetic engineering in pines forestry. The gymnosperm softwood, such as pine wood, contains roughly 42% cellulose, 27% hemicellulose and 28% lignin [31] (Table 1). In conifers, lignin consists of only two types of phenylpropane units, the "H" and the "G" units, thus, lacking the third "S" type which naturally exists in angiosperm hardwood [59]. It was reported that the F5H homolog, which is a crucial gene for the biosynthesis of S-units, was not identified in the pine genome [184, 194]. Therefore, engineering the syringyl lignin pathway into conifers is novel,

making the coniferous wood chemistry similar to that of angiosperms [195]. With further genetic alteration of the S/G ratio at the expense of the G-units, the resultant pine wood is expected to be improved towards better biofuel production. Earlier studies [196, 197] which dealt with a natural mutation in the *cad* gene that involves the lignin biosynthetic pathway, revealed that mutated loblolly pine trees showed significant, but variable increase in growth and wood volume, depending on the age and genetic backgrounds of the tested populations. Recently, novel GM loblolly pine varieties with altered lignin and cell wall composition are being optimized, in a xylem specific manner, via manipulation of glucommann (*LpGluM*) genes, and three genes encoding transcription factor NAC (for *NAM*, *ATAF1/2* and *CUC2*) domain proteins (LpNAC1, LpNAC2, and LpNAC3) that are implicated in the regulation of plant polysaccharides [198, 199]. The ultimate goal is to improve the physical and chemical properties of wood for efficient deconstruction and sugar release for commercial cellulosic ethanol production. The outcomes of such worthy efforts can provide tree breeders with insights into the ideal wood properties, and breeding guidelines for future pine tree stock development.

3 Management of potential transgene flow risks of GM trees

It is likely that commercialization of GM tree plantations is in the process of being approved in some countries within Asia, North and South America, e.g. GM poplar grown in China, and transgenic eucalyptus being tested in Brazil [162], leaving Europe lagging behind [200]. GM trees still need to be assessed, approved and then de-regulated before the release and deployment in commercial forestry (see review by [201]). Biosafety and risk assessment strategies for transgene confinement in the engineered trees, as well as mitigation of potential risks that may arise due to possible transgene flow into compatible and closely related natural populations, need to be implemented to avoid uncertain ecological consequences, and to satisfy regulatory requirements and public acceptance. In general, transgenic trees might remain in the ecosystem for several years, dispersing their propagules recurrently for many years before harvesting, and thus, their potential impact on natural plants, animals and soil would be longer than that of annually-grown bioenergy crops [162, 202]. Therefore, biosafety risk-assessments and regulations governing engineered trees would be very difficult and extremely costly due to the long period of time necessary to monitor environmental effects, including: gene stability, pollen and seed dispersal and impact on other elements of the ecosystem [203].

Not all transgenes pose potential risk in terms of gene escape and non-target impacts. Traits for bioenergy biomass production such as wood quality and altered lignin might be considered low risk [10, 201, 204]. In contrast, transgenes that may improve competitive fitness or increase invasiveness of engineered trees (e.g. herbicide and pest resistance traits) especially non-native tree species outside managed plantations, are considered for risk evaluation. Thus, transgenesis assessments would be made on a case-specific basis, and mainly based on the different levels of potential risks, which depend on the biology of the host tree species, the characteristics of the introduced trait, its impact on growth fitness, and non-target impacts [201, 204]. Several containment and mitigation approaches have been and are being developed to deal with risky transgene flow from transgenic plants, including GM perennials [205–207]. Transgene bioconfinement strategies (reviewed in [208]) mainly rely on regenerating trees with delayed flowering or modified to be reproductive sterile without affecting other functions in the engineered plants [205, 207]. Flowering delay could be achieved by modifying the expression of genes that promote vegetative growth or repress the transition to reproductive growth. It could be applied in short-rotation coppice systems such as poplar and willow species, as such trees can be harvested before the onset of flowering, thus avoiding potential risk of transgene flow via pollen or seed dispersal from GM plantations. On the other hand, floral development modifications, and pollen and seed sterility are potential tools for transgene confinement, including: floral cell ablation through floral-specific expression of a cytotoxin gene [209, 210]; transgene excision from gametes [e.g. 211, 212]; or silencing one or more native genes essential for reproduction at DNA, RNA or protein levels [205, 207]. Nevertheless, induced-sterility containment techniques are not absolute and, thus, transgene leakage could be unavoidable [202]. Thus, a complementary failsafe approach of transgene mitigation (TM) might be significant at this level. This approach can be employed using TM genes that can increase value in managed environments, but reduce competitive fitness in the wild [213]. In this regard, TM traits such as semidwarfism, flower ablation, or prevention of pollen formation could be tightly linked to herbicide resistance or other primary feedstock traits, thus do not segregate in the transgenic recipients. Furthermore, as commercial forestry is increasingly using conventional vegetative propagation or in vitro somatic embryogenesis, then, the control of pollen, flower drop, and fruit set

would impede transgene escape by pollen or seed to non-target plant species.

4 Concluding remarks

Biotech improvements are necessary to make purpose-grown trees a sustainable and economical feedstock option for efficient production of cellulosic ethanol and other forms of bioenergy. In order to re-shape the future of tree-based energy supply, scientific breakthroughs through basic research on genes and pathways involved in cell wall biosynthesis, plant development and interactions with the surrounding environment, should coincide with investments of novel tools and emerging biotechnologies. Most of wood production and property traits such as growth rate, stem and wood quality, and adaptability are under polygenic control fashion, rather than a single gene control. Therefore, it is necessary to increase our knowledge of the overall molecular control of the desired traits engineered into promising tree species for significant energy production.

Moreover, collaborative approaches of advanced breeding, genetics, and "omics" technologies, such as those carried out on *Populus, Eucalyptus* and *Pinus* species, including the on-going isolation and characterization of novel promoters, transcription factors and genes, are expected to open up new avenues for domestication and transgenesis of purpose-grown and short-rotation dedicated trees for better applications worldwide. Still, many approaches are run by private sectors or government research institutes, which preclude or prevent dissemination of some scientific knowledge into the public domain.

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