Recent advances in forest tree biotechnology

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Abstract Forest trees produce an important feedstock, wood. Forest tree breeding programs have been traditionally carried out by selecting elite trees to enhance productivity and processability. Recently, however, a biotechnological approach has attracted much attention because it enables efficient and versatile improvement of forest trees. In the last decade, forest tree biotechnology has considerably progressed: genomic sequences of several forest tree species have been decoded, efficient Agrobacterium-mediated genetic transformation and regeneration systems have been established in a number of forest tree species, and many reports have been published on the metabolic engineering of a major wood component, lignin, in forest trees. However, in contrast to the metabolic engineering of lignin, the metabolic engineering of cellulose and hemicelluloses in forest trees awaits further development. The detrimental effects on tree growth are often concomitant with the metabolic engineering of wood components. To mitigate such effects, fine-tuned regulation of transgene expression, and the production of value-added products may be targeted in future forest tree biotechnology.

Key words: Genomic sequencing, transgenic technology, metabolic engineering, wood.

Forest trees are important for the environment of the earth as well as for human life. Forest trees comprise about 70–90% of terrestrial biomass, which greatly impacts the carbon, water, and oxygen cycles in the atmosphere (Houghton et al. 2009). In addition, forest trees accumulate huge amounts of wood in their trunks. Human beings utilize wood as lumber, fuel, and feedstock for pulp and paper. Recently wood has also attracted attention as feedstock for bioenergy as a carbon-neutral renewable resource (Sannigrahi et al. 2010).

Because forest trees produce important feedstock, wood, forest tree breeding programs have been traditionally carried out by selecting elite trees. However, due to the long life cycles, long generation times, and the late sexual maturity of forest trees, traditional tree breeding programs require very long time intervals. Furthermore, genes responsible for versatile demands in terms of commercially important traits are often not available within the gene populations of the target tree species. Thus, these necessitate better tree improvement programs in which modern biotechnology plays an important role (Umezawa et al. 2008).

Thus far, many reports of functional genomics and metabolic engineering have been published about the poplar species (Ye et al. 2011). Recent advances in the massive parallel sequencing of the genome and the transcriptome have been boosting such research in various forest tree species (Neale and Kremer 2011). In this review, we focus on the most recent advances of forest tree biotechnology including genomic sequencing, transgenic technology, and the metabolic engineering of wood components in forest trees as a way to benefit researchers in future biotechnological research for tree improvement.

Genomic sequencing

The genome sequencing of a tree species was reported in 2006 for the first time (Tuskan et al. 2006). They sequenced the genome of a female strain of Populus trichocarpa “Nisqually-1”. The total number of coding genes is 41,335, and the genome size is approximately 423 Mb according to the P. trichocarpa genome assembly ver. 3.

A decade ago, genome sequencing of a plant...
species was performed through a large-scaled project (International Rice Genome Sequencing Project 2005; Tuskan et al. 2006). Because the next-generation sequencer enabled cost-effective massive parallel sequencing, many genomic sequencing projects have been completed or are currently underway. For example, two genomes of the Eucalyptus species have been sequenced in Japan and the US: E. camaldulensis (Hirakawa et al. 2011) and E. grandis (Myburg et al. 2011). More recently, a draft assembly of the 20-Gb Norway spruce (Picea abies) genome sequence has been reported. The genome size of the Norway spruce is more than 100 times bigger than that of Arabidopsis thaliana, but the number of well-supported genes (28,354) is similar to that of A. thaliana (Nystedt et al. 2013). In addition, whole genomic sequences from the dwarf birch (Betula nana) (Wang et al. 2013) and fruit trees such as the grapevine (Vitis vinifera) (The French-Italian Public Consortium for Grapevine Genome Characterization 2007), Japanese apricot (Prunus mume) (Zhang et al. 2012), peach (Prunus persica) (The International Peach Genome Initiative 2013), pear (Pyrus bretschneideri) (Wu et al. 2013), apple (Marus × domestica) (Velasco et al. 2010), papaya (Carica papaya) (Ming et al. 2008), cocoa tree (Theobroma cacao) (Argout et al. 2011), and Jatropha curcas (Sato et al. 2011) were decoded. These genomic resources will help open new genomic avenues for forest tree biotechnology.

Transgenic technology

Agrobacterium-mediated genetic transformation and regeneration systems are now available in a number of forest tree species (Table 1). Because of the ease of transformation and regeneration, aspen and its hybrids (e.g. Populus tremula × tremuloides) have been widely used for research purposes. On the other hand, an efficient transformation and regeneration system for P. trichocarpa "Nisqually-1" has been desired for the functional genomics of Populus species because the genomic sequence has already been decoded (Tuskan et al. 2006). However, genotype Nisqually-1 was known to be recalcitrant for transformation and regeneration. Song et al. (2006) reported a highly efficient transformation and regeneration system for this genotype, which can be used for functional genomics in the poplar.

Table 1. Agrobacterium-mediated transgenic system for forest trees.

<table>
<thead>
<tr>
<th>Family</th>
<th>Scientific name</th>
<th>Explant</th>
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<th>Binary vector</th>
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Metabolic engineering of wood components

Currently, wood produced by industrial forest trees has been utilized mainly as feedstock for pulp and timber industries. In these industries, the chemical and physical properties of wood are of course important factors to be considered. Wood properties are significantly affected by the properties of the thick cell walls. The cell walls are mainly composed of cellulose, hemicelluloses (glucuronoxylan and glucomannan), and lignin. Therefore, the quantity, structure, and distribution of these components influence properties such as strength, fiber quality, and pulp yield. Furthermore, fast-growing trees such as the poplar and eucalypt have become attractive recently as feedstock for cellulosic biorefinery (Sannigrahi et al. 2010). Also, increasing the yield of fermentable sugar from the feedstock would benefit the growing biorefinery industries. The improvement of saccharification could be achieved by the alteration of the wood components. Thus, the metabolic engineering of the lignin, cellulose, and hemicelluloses in forest trees is one of the hottest topics in forest tree biotechnology.

Metabolic engineering of lignin

Lignin is a natural aromatic polymer generated by the radical coupling of monolignols (4-hydroxycinnamyl alcohols) (Umezawa 2010; Vanholme et al. 2012). To date, a principal biosynthetic pathway towards lignin has been proposed (Figure 1). In this pathway, phenylalanine is deaminated by phenylalanine ammonia-lyase (PAL) to produce cinnamic acid. Cinnamic acid is converted to p-coumaric acid by cinnamic acid 4-hydroxylase (C4H). p-Coumaric acid is then converted by 4-coumarate:CoA ligase (4CL) to p-coumaroyl-CoA. p-Coumaroyl-CoA couples with shikimic acid to produce p-coumaroyl shikimate by a hydroxycinnamoyltransferase (HCT). p-Coumaroyl shikimate is next converted to caffeoyl shikimate by p-coumarate 3-hydroxylase (C3H). Caffeoyl shikimate is further hydrolyzed by HCT or recently identified caffeoyl shikimate esterase (CSE) (Vanholme et al. 2013), and then caffeoyl-CoA or caffeic acid is produced. Recently, an alternative pathway via a direct conversion from cinnamic acid to caffeic acid via p-coumaric acid by a C4H–C3H complex has been reported (Chen et al. 2011). In this shunt, the resulting caffeic acid is activated by 4CL to yield caffeoyl-CoA. In the biosynthetic pathway towards a major monolignol coniferyl alcohol, caffeoyl-CoA is

![Figure 1. The proposed principal biosynthetic pathway towards lignin. 4CL, 4-coumarate:CoA ligase; C3H, 4-coumarate 3-hydroxylase; 4H, cinnamate 4-hydroxylase; 4H–C3H, an enzyme complex composed of 4H and C3H; CAD, cinnamyl alcohol dehydrogenase; CALd5H, coniferaldehyde 5-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamyl-CoA reductase; COMT, caffeic acid/5-hydroxyconiferaldehyde O-methyltransferase; CSE, caffeoyl shikimate esterase; HCT, hydroxycinnamoyl-CoA-quinate/shikimate hydroxycinnamoyltransferase; PAL, phenylalanine ammonia-lyase; SAD, sinapyl alcohol dehydrogenase.](image-url)
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revealed that et al. (2011). Using microscopic analysis, Kitin et al. conductivity compared with that of the control (Voelker significantly decreased the xylem-specific water in the field showing substantial lignin reductions pretreatment. amenable to enzymatic hydrolysis with or without Populus trichocarpa showed more black cottonwood (4CL reported that the saccharification efficiencies were not associated deformed. However, acetyl bromide lignin and molecular beam mass spectroscopy-based lignin contents in the brown wood were similar to those of the control, and the saccharification efficiencies were not associated with the lignin reduction. By contrast, Min et al. (2012) reported that 4CL-downregulated low-lignin lines of black cottonwood (Populus trichocarpa) showed more amenable to enzymatic hydrolysis with or without pretreatment.

Some of antisense 4CL-downregulated poplar grown in the field showing substantial lignin reductions significantly decreased the xylem-specific water conductivity compared with that of the control (Voelker et al. 2011). Using microscopic analysis, Kitin et al. (2010) revealed that 4CL-downregulated low-lignin hybrid white poplar contained areas of nonconductive, brown xylem with patches of collapsed cells and patches of noncollapsed cells filled with phenolics. In contrast, phenolics and nonconductive vessels were rarely observed in normal colored wood of the low-lignin trees. Moreover, many of the vessels in the nonconductive xylem were blocked with tyloses. The authors concluded that the reduced transport efficiency of the transgenic low-lignin xylem was largely caused by blockages from tyloses and phenolic deposits within vessels rather than by xylem collapse. On the other hand, RNA interference (RNAi) suppression of 4CL driven by a Pinus radiata CAD promoter resulted in dwarfed plants with a “bonsai tree-like” appearance in P. radiata (Wagner et al. 2009). The tracheids were occasionally deformed and ununiformly lignified, and circumferential bands of axial parenchyma were developed. In the most suppressed lines, 36 to 50% of lignin was reduced based on acetyl bromide-soluble lignin assay and nuclear magnetic resonance (NMR) analysis.

Coleman et al. (2008) reported the downregulation of C3H in the hybrid poplar by RNAi. The acid insoluble lignin content of the most strongly repressed line was almost reduced by half, and the significant shift in lignin monomer composition was observed, favoring the generation of p-hydroxyphenyl units at the expense of guaiacyl units while the proportion of syringyl moieties remained constant. Furthermore, suppression of C3H resulted in the accumulation of substantial pools of 1-O-p-coumaryl-β-d-glucoside and other phenylpropanoid glucosides. Later, Ralph et al. (2012) confirmed the alteration of lignin monomer composition in the C3H-downregulated poplar using two-dimensional (2D) NMR methods.

In CCoAOMT-downregulated poplar lines, an approximately 40% reduction in Klon lignin content in the most repressed line has been reported, but no significant effect on plant growth and morphology by CCoAOMT-downregulation (Zhong et al. 2000). On the other hand, suppressed lines showed a 12% reduction in Klon lignin content and an 11% increased syringyl/guaiacyl (S/G) ratio in the noncondensed lignin fraction (Meyermans et al. 2000).

The significant incorporation of an unusual lignin monomer, ferulic acid, into lignin was found in CCR-downregulated poplars (Leplé et al. 2007). The CCR-downregulation was associated with up to 50% reduced lignin content and an orange-brown, often patchy, coloration of the outer xylem. Lignin was relatively more reduced in syringyl than in guaiacyl units. Ferulic acid was incorporated into the lignin via ether bonds, which was independently evidenced by thioacidolysis and NMR. Chemical pulping of wood derived from 5-year-old, field-grown transgenic lines revealed improved pulping characteristics, but growth was affected in all transgenic lines tested. CCR was also downregulated in the Norway spruce (Wadenbäck et al. 2008). The lignin reduction was up to 8%, and the content of p-hydroxyphenyl lignin was reduced compared to the control. Similarly to the CCR-downregulation in poplar (Leplé et al. 2007), chemical pulping characteristics were improved.

It is well known that CAD-downregulation results in
the increase of hydroxycinnamaldehyde units in lignin (Koshiba et al. 2013b). The red purple coloration in the CAD-downregulated tobacco xylem has been attributed to the incorporation of hydroxycinnamaldehydes into lignin (Hibino et al. 1995). Baucher et al. (1996) reported downregulation of CAD in hybrid poplar by antisense and cosuppression strategies. No significant change in lignin content and composition (S/G ratio) was observed in the downregulated CAD poplar. Later, Lapierre et al. (1999) tested the growth and reactivity to Kraft pulping using 2-year-old CAD-downregulated hybrid poplars. The transgenic poplar showed growth similar to the control trees. The Klasson lignin was slightly reduced, but the increased proportion of free phenolic groups in the lignin facilitated lignin solubilization and fragmentation during Kraft pulping.

CAld5H-upregulation driven by an Arabidopsis C4H (AtC4H) promoter in poplar displayed an enhanced S/G ratio up to about 5.7 (Franke et al. 2000). Using the P. tremuloides 4CL1 promoter, CAld5H was overexpressed in P. tremula, which resulted in the S/G ratio up to 5.5 (Li et al. 2003). The AtC4H::F5H transgenic poplar wood was later subjected to pulping (Huntley et al. 2003) and 2D NMR (Stewart et al. 2009) analyses. The lignin structure of transgenics was linear and occupied by almost syringyl units (up to 97.5%). The lignin displayed a lower degree of polymerization than that of the control (Stewart et al. 2009).

COMT (or CAOMT) is first named as caffeic acid O-methyltransferase, but it has also been named 5-hydroxyconiferaldehyde O-methyltransferase (CAldOMT) (Koshiba et al. 2013a) because it was found that 5-O-methylation activity towards 5-hydroxyconiferaldehyde was competitively prominent (Osakabe et al. 1999). Here we use COMT as an abbreviation of caffeic acid/5-hydroxyconiferaldehyde O-methyltransferase (Shi et al. 2010). Sense and antisense COMT from P. trichocarpa × P. deltoides were individually overexpressed under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter. In severely repressed transgenic lines with an antisense construct, the S/G ratio was reduced by sixfold, and 5-hydroxyguaiaacyl residue was detected among the thioacidolysis products. Furthermore, the wood of transgenic poplar colored in pale rose. However, lignin content of the transgenic poplars was similar to that of the controls (van Doorsselaere et al. 1995). On the other hand, by using COMT from P. tremuloides, COMT-cosuppressed lines were produced in P. tremuloides under the control of a double CaMV35S promoter. In some transgenic lines, the enzymatic activity was significantly suppressed in xylem, but significantly increased in leaf and sclerenchyma tissues compared to the control, indicating that the occurrence of sense cosuppression depends on the degree of sequence homology and endogene expression. Characterization of the lignins isolated from the cosuppressed lines revealed that a high amount of coniferaldehyde is the origin of the red-brown coloration (Tsai et al. 1998). Later, Jouanin et al. (2000) produced COMT-cosuppressed lines driven by double CaMV35S promoter. In the severely cosuppressed lines, COMT activity was almost zero, and 17% of lignin was decreased. Lignin structure was found to be strongly altered, with a two times higher content in condensed bonds, an almost complete lack of syringyl units, and the incorporation of 5-hydroxyguaiaacyl units. Kraft-pulping assays revealed that pulp yield from the cosuppressed lines was 10% improved compared to the control, but this positive effect was severely counterbalanced by a detrimentally high kappa number diagnostic for a higher residual lignin content in the pulp.

Metabolic engineering of cellulose and hemicelluloses

In contrast to the metabolic engineering of lignin, the examples for cellulose and hemicelluloses are not many in forest trees.

Cellulose is synthesized from uridinediphosphate (UDP)-glucose by the cellulose synthase complex on the plasma membrane. The catalytic subunits are believed to be encoded by cellulose synthase (CesA) genes. Previous study revealed that at least three types of CesAs are required for normal cellulose biosynthesis during either primary or secondary wall formation. Mutations in any one of CesAs disrupt cellulose synthesis, indicating the non-redundant function of members of the different subclass members in Arabidopsis (Joshi et al. 2011; Somerville 2006). In secondary wall formation, three distinct CesAs (AtCesA4, AtCesA7, and AtCesA8) are required in Arabidopsis. To date, three distinct CesAs orthologous to secondary wall-related AtCesAs were cloned from aspen and characterized (Wu et al. 2000). In sense cosuppression of a poplar CesA (PtdCesA8) orthologous to AtCesA8, secondary xylem of transgenic aspen contained as little as 10% cellulose normalized to dry weight compared to 41% cellulose typically found in normal aspen wood. This massive reduction in cellulose was accompanied by proportional increases in lignin (35%) and non-cellulosic polysaccharides (55%) compared to the 22% lignin and 36% non-cellulosic polysaccharides in control plants. The transgenic stems produced deformed vessels and contained greatly reduced amounts of crystalline cellulose (Joshi et al. 2011).

Glucuronoxylan is a major hemicellulose of angiosperm wood. The linear polysaccharide is composed entirely of 1,4-linked β-D-xylene and is partially substituted by 4-O-methyl-α-D-glucuronic acid through α-1,2-glycosidic linkages. A portion of the backbone is acetylated at either C-2 or C-3 of
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Recent advances in forest tree biotechnology reported the simultaneous downregulation of \textit{PtrGT8D1} and \textit{PoGT47C}-downregulated lines leads to an increased glucuronoxylan content in 1 M KOH extract but no change in other cell wall sugars including mannose, galactose, arabinose, and rhamnose. Immunodetection revealed that glucuronoxylan in the wood of \textit{PoGT47C}-downregulated lines was reduced. Reduction in glucuronoxylan in the wood of \textit{PoGT47C}-downregulated lines leads to an increased digestibility of wood by cellulases. Li et al. (2011) reported the simultaneous downregulation of \textit{PtrGT8D1} and \textit{PtrGT8D2} orthologous to \textit{Arabidopsis} \textit{IRX8} in \textit{P. trichocarpa}. The transgenic lines exhibited 29–36% reduction in stem wood xylan content. Xylan reduction had essentially no effect on cellulose quantity but caused an 11–25% increase in lignin. Stem modulus of elasticity and modulus of rupture were reduced by 17–29% and 16–23% respectively, and were positively correlated with xylan content but negatively correlated with lignin quantity, suggesting that xylan may be a more important factor than lignin in affecting the stiffness and fracture strength of wood.

Xyloglucan is the most abundant hemicellulose in the primary walls of angiosperms (Pauly et al. 2013), tightly tethering cellulose microfibrils noncovalently (Hayashi, 1989). This xyloglucan-cellulose framework is modified by xyloglucan \textit{endo}-transglycosylases (XETs) (Nishikubo et al. 2011). Overexpressing xyloglucanase resulted in growth enhancement and cellulose accumulation (Park et al. 2004), and acceleration of enzymatic digestibility of wood cellulose in poplar (Kaida et al. 2009) and wood polysaccharide in \textit{Acacia mangium} (Kaku et al. 2011). The amount of xyloglucan is little in wood, but XET is actively expressed in the wood forming tissues of aspen (Nishikubo et al. 2011). These results suggest that xyloglucan plays an important role in wood formation (Hayashi and Kaida 2011; Mellerowicz et al. 2008).

**Conclusion and future prospectives**

Recent advances of DNA sequencing using a next-generation sequencer is accelerating the genome sequencing project of forest trees. In addition to fast-growing hardwood tree species such as \textit{Populus} and \textit{Eucalyptus}, softwoods such as pine and spruce, whose genome size is very large, have also become targets. \textit{Agrobacterium}-mediated transformation and regeneration were achieved in a number of important hardwood and softwood species. By coupling genomic resources with transgenic technology, gene characterization and metabolic engineering will be accelerated in species other than poplar.

In the field of metabolic engineering of wood, lignin biosynthetic engineering has progressed considerably because the genes that encode enzymes involved in lignin biosynthesis have been almost identified in poplar. As a result, it is now technically possible to achieve more than a 50% reduction of lignin content in the xylem of poplar (Kitin et al. 2010). Simultaneously, however, such reduction in xylem occasionally causes detrimental effects on the growth of the transgenic trees. In the next stage, we hope to target the combinatorial modification of lignin using multiple upregulation and/or downregulation of the gene involved in lignin biosynthesis. Furthermore, to mitigate the detrimental effects caused by low-lignin in vessels, technology to maintain lignin in vessels and to reduce lignin in fibers in the stem xylem must be developed. As such, a recent report of fiber-specific reduction of lignin and increase of cellulose and xylan in \textit{Arabidopsis} inflorescent stems is remarkable (Yang et al. 2013). The further introduction of a metabolic pathway to utilize the surplus phenolic metabolites produced by lignin reduction should be instrumental in efficiently utilizing such engineered wood.

In contrast to lignin biosynthetic research, much remains to be elucidated in the identification and characterization of the genes involved in cellulose and glucuronoxylan biosynthesis. As gene identification and characterization progress, the fine-tuned metabolic engineering of cellulose and glucuronoxylan biosynthesis will be realized.

**References**


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