

Forest tree biotechnology

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The past year has seen the fruits of biotechnological manipulation of forest trees approach commercial application. Advances in somatic embryogenesis have brought mass clonal propagation of the top commercial trees closer to reality, and efficient gene transfer systems have been developed for a number of conifers and hardwoods. Radical alterations in the quantity and quality of lignin in wood have been shown to be possible in softwoods and hardwoods through identification of naturally occurring mutants, as well as by engineering the lignin biosynthetic pathway with transgenes. The potential environmental and social impacts of the release of transgenic trees have become an increasingly contentious issue that will require more attention if we are to use these technologies to their full advantage.

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Abbreviations

CAD cinnamyl alcohol dehydrogenase

EST expressed sequence tag

GUS β -glucuronidase

OMT O-methyltransferase

Introduction

Forest trees have undergone relatively little domestication, consequently biotechnology could potentially have a greater impact on forestry and forest products than it has had on agronomic crops. Biotechnological approaches, such as *in vitro* propagation, gene transfer and marker-assisted breeding, have done much to bring the genetic improvement of forest trees to a level of sophistication comparable with that routinely used for agronomic species. Some of the most problematic barriers to genetic improvement of forest trees, such as their large size and long breeding cycles, can be circumvented by the application of these new techniques. However, other features inherent to the biology of forest trees (e.g. the large genome sizes of pines and the recalcitrance of tissues from mature trees to *in vitro* manipulation) continue to present challenges to researchers. Although the biotechnological improvement of forest trees spans a broad range of topics, this review will highlight recent progress in mass clonal propagation of commercial species, new gene transfer systems, genomics and molecular breeding, lignin deposition/wood formation, and considerations for the environmental release of transgenic trees.

In vitro propagation

The primary applied goal of *in vitro* culture of forest trees has always been mass clonal propagation of the most desirable

genotypes, although more recently the provision of target material for gene transfer has assumed prominence. Although the first plantlets from forest tree tissue culture were produced in the 1960s via adventitious shoots, the forest industry has more recently focused research efforts on *in vitro* propagation via somatic embryogenesis, defined as the formation of an embryo from a cell other than a gamete or the product of gametic fusion. This approach appears to have several advantages over other *in vitro* propagation systems, including its potentially high multiplication rates, potential for scale-up and delivery via bioreactor and synthetic seed technologies, and the fact that embryogenic cultures make suitable target tissue for gene transfer [1]. Embryogenic cultures have been generated for most commercially important conifers and hardwoods. For the most part, however, even the best of these systems lack commercial viability for two reasons: firstly, low frequency of regeneration for many of the most desirable clones; and secondly, unproven genotypes, as most starting material for the cultures is derived from seeds or seedlings.

Although much of the work in conifer embryogenesis has been conducted by forest products company scientists, and is thus mostly disclosed only in patents, some recent progress has been reported in the scientific literature. Research with spruces and pines, in particular, has focused on improving somatic embryo quality, which should lead to higher plantlet production rates. Starting from protocols already patented by industry researchers, treatments with abscisic acid, polyethylene glycol and maltose were confirmed to promote the highest production of mature *Pinus taeda* (loblolly pine) somatic embryos [2,3]. Gelling agent concentration was shown to have an impact on both initiation of pine embryogenic cultures and on maturation of somatic embryos. Whereas a relatively low concentration (2 mg/l) of gellan gum was optimal for initiation of embryogenic *P. taeda* cultures [4], a fivefold higher level of gellan gum aided maturation and germination of somatic embryos of *Pinus strobus* [5], *Pinus sylvestris* and *Pinus pinaster* [6]. Another surprising finding with *P. sylvestris* cultures was that somatic embryos developed and matured spontaneously with no exposure to exogenous plant growth regulators.

Although encouraging, this progress with somatic embryogenesis is still the result of empirical experimentation, which has long been a hallmark of plant tissue culture research. Thus, it is highly significant that the past year has seen the application of a genomics approach to improving regeneration from forest tree cultures in a more systematic manner by understanding the developmental changes occurring *in vitro* at the gene expression level [7].

The past few years have also seen encouraging progress with regard to the propagation of proven genotypes via

somatic embryogenesis from tissues of mature trees. Embryogenic cultures of both *Pinus radiata* (radiata pine) [8•] and *Picea abies* (Norway spruce; M Pâques, J Bercetche, L Harvengt, abstract 4, Joint Meeting of the IUFRO Working Parties 2.04-07 and 2.04-06 Somatic Cell Genetics and Molecular Genetics of Trees, 12–16 August 1997, Quebec City) were initiated from trees up to 20 and 25 years old, respectively. Although details of the protocols were not reported, both studies presented evidence that the material underwent rejuvenation in the process. For hardwood species, floral and inflorescence tissues, in particular, have proven to be useful explants for initiation of embryogenic cultures from mature trees [9]. As *Eucalyptus* species have risen in economic status, an increasing number of reports have appeared on *in vitro* propagation of members of this genus. Most recently, regeneration of *Eucalyptus nitens*, *Eucalyptus globulus* and a *Eucalyptus grandis* × *Eucalyptus urophylla* hybrid was reported via adventitious shoot production from seedling explants [10,11].

Even with systems for *in vitro* propagation from mature tree tissues, *in vitro* cultures need to be maintained for years while propagules derived from them are tested in the field. Serial transfer of cultures over long periods is problematic due to such factors as labor costs, risk of contamination, loss of regeneration potential, and somaclonal variation. With regard to somaclonal variation, a recent test of 87 embryogenic *Picea mariana* and *Picea glauca* clones from which plantlets were regenerated over a five year period indicated that clonal line and time in culture were the two most important sources of genetic instability for the cultures [12]. Fortunately, *in vitro* cultures, and embryogenic cultures in particular, have proven amenable to cryostorage. A set of twelve *P. glauca* cultures cryopreserved for 3–4 years and repropagated by somatic embryogenesis demonstrated general stability with regard to morphology, *in vitro* development, *ex vitro* survival, and growth characteristics [13]. Random amplified polymorphic DNA (RAPD) analysis of another set of *P. glauca* embryogenic clones, however, detected a low level of somaclonal variation within two months following re-establishment from cryostorage [14].

Development of gene transfer technology

While the first transgenic conifers were generated in the early 1990s, reports of stable transformation of pine, in particular, were absent until Walter *et al.* [15••] reported regeneration of transformed *P. radiata* plantlets from microprojectile-bombarded embryogenic cultures. Using the *uidA* gene encoding β -glucuronidase (GUS) under the control of either a double cauliflower mosaic virus (CaMV) 35S promoter or an artificial Emu promoter, more than 150 transgenic radiata pine plantlets were produced from 20 independent transformation events using four different embryogenic clones. Microprojectile bombardment of embryogenic cultures has also been used to produce transgenic *P. mariana* [16] and *Larix laricina* [17] plantlets. In addition to GUS, green fluorescent protein (GFP) has

recently proven to be a useful reporter for transformation studies with both conifers and hardwoods [18].

Agrobacterium-Ti plasmid-mediated transformation was recently achieved for the first time in both *Pinus* and *Picea*. Similar to previous reports for other species, adding extra copies of genes involved in *Agrobacterium* virulence and T-DNA transfer (*virG*, *virB*) to disarmed strains of *Agrobacterium tumefaciens*, increased transformation efficiencies for embryogenic *P. abies* cultures 1000-fold, as determined by GUS expression. A 10-fold increase in transient GUS expression was obtained using the same approach with embryogenic *P. taeda* cultures [19••]. Co-cultivation of *P. strobus* embryogenic tissue with *A. tumefaciens* carrying a 35S-35S-AMVgus::nptII fusion also resulted in the regeneration of stably transformed somatic embryos [20].

As with conifers, the first report of stably transformed hardwood trees is over a decade old, yet additional progress in transformation of hardwood trees has remained concentrated to a few genera. *Agrobacterium*-mediated protocols have been developed for most *Populus* species and hybrids [21]. Transformation of one of the top commercial hardwood genera, *Eucalyptus*, was recently achieved via co-cultivation of seedling hypocotyls with *A. tumefaciens* [22]. One promising commercial application of transgenic hardwoods is for phytoremediation, the use of plants to stabilize, reduce or detoxify pollutants. *Liriodendron tulipifera* trees transformed with a modified bacterial mercuric ion reductase gene were demonstrated to survive on normally toxic levels of ionic mercury *in vitro* by reducing it to less toxic metallic mercury [23•].

Genomics and molecular breeding

Two public-domain projects focused on gene discovery in commercial forest tree species have begun to make information available to the wider research community. A pilot study to characterize expressed sequence tags (ESTs) associated with wood formation in loblolly pine identified nearly 1100 gene products [24]. This project has been greatly expanded and as of mid-October 1999 over 5500 EST sequences were available in a database that may be accessed via the Internet (<http://www.cbc.umn.edu/ResearchProjects/Pine/DOE.pine/index.html>). A similar project studying wood formation in *Populus* compared the expression of more than 5600 ESTs in xylem and phloem tissues [25]. An Internet-accessible database also exists for the *Populus* ESTs (<http://kiev.physchem.kth.se/PopulusDB/>). In an excellent example of how such ESTs may be employed to further our understanding of tree biology, an array of several hundred ESTs from loblolly pine was used to identify differences in the gene expression patterns that occur during the development of zygotic and somatic loblolly pine embryos [7•]. Such DNA arrays will provide a powerful tool for rapid optimization of *in vitro* culturing techniques for production of high-quality somatic embryos by helping to identify *in vitro* conditions promoting development of zygotic-like qualities.

In the area of molecular markers, initial efforts at comparative mapping of the loblolly and radiata pine genomes found that a significant number of markers developed in one species could effectively be used in the other, suggesting that the development of a 'generic' pine genetic map might be possible [26]. Such a generic genetic map could eventually speed identification and isolation of useful genes from less-studied pine species. New high-throughput techniques for quantifying wood properties are being used in conjunction with genetic maps to identify quantitative trait loci of particular commercial interest [27*].

Lignin deposition and wood formation

Considerable excitement has developed recently as several groups have shown that by manipulating the expression of genes in the phenylpropanoid pathway, lignin quantity and composition can be affected much more radically than was previously expected [28]. Baucher *et al.* [29] have written an excellent review that will serve to update the reader interested in a more detailed understanding of lignin biosynthesis and manipulation. Although lignins having unusual subunit compositions were recognized long ago in the brown-midrib mutants of monocots [30], the first strong evidence for what might be possible for lignin modification in trees came from the identification of a null allele for the cinnamyl alcohol dehydrogenase (CAD) gene in loblolly pine [31,32]. Wood from a loblolly pine that was homozygous for this recessive allele was red-brown in color and contained elevated levels of soluble phenolic compounds; however, the total lignin content was little changed. The lignin polymer from this tree also contained several unusual substituents. Subsequent studies have shown that the lignin is more easily removed in mild alkali treatments and soda pulping, suggesting that alteration of this gene may hold promise for commercial pulping [33]. Transgenic poplars in which CAD activity was reduced by up to 70% through the expression of an antisense CAD gene showed a modest reduction in Klason lignin and superior pulping characteristics [34].

Angiosperm lignins are more easily extracted from wood during the pulping process by virtue of their less condensed nature resulting from the incorporation of syringyl residues. This has led to substantial interest in the possibilities for engineering gymnosperms to express genes that would produce sinapyl alcohol in addition to coniferyl alcohol. Angiosperms appear to employ two different classes of *O*-methyltransferases (OMTs) in the biosynthesis of lignin precursors; one class acts predominantly on the CoA-thioesters (caffeoyl coenzyme A 3-*O*-methyltransferase [CCoAOMT]) and the other class on the free acid (caffeic acid *O*-methyltransferase [CAOMT]) forms of the monolignol precursors [35]. Suppression of CAOMT was shown to increase the coniferaldehyde content of the lignin in transgenic quaking aspen [36], and it made lignin more difficult to extract from transgenic poplar [34]. Suppression of CCoAOMT increased the coniferaldehyde content of

the lignin in transgenic tobacco, but this change also reduced the quantity of lignin in these plants [35]. An appreciation of the regulatory potential of using CoA-thioesters as substrates for the biosynthesis of lignin precursors has also altered our current view of the 5-hydroxylation reaction that immediately precedes the methylation step. Thus, two laboratories working with cytochrome P450 monooxygenases from *Arabidopsis* [37] and *Liquidambar styraciflua* (sweetgum) [38] showed that feruloyl-CoA, rather than ferulic acid, is the most likely substrate for this critical reaction in the biosynthesis of sinapyl alcohol. Since the incorporation of sinapyl alcohol into angiosperm lignin is considered one of the major factors enabling easier pulping of hardwoods, there has been considerable interest in the potential for introducing the genes for sinapyl alcohol biosynthesis into gymnosperms. It remains to be seen, however, whether expression of the appropriate angiosperm hydroxylase and OMT genes in a gymnosperm will be sufficient to make softwoods easier to pulp.

Arguably the news that has stimulated the greatest interest recently came in a report by Hu *et al.* [39**] which showed that reduced expression of the 4-coumarate:CoA ligase (4CL) gene in transgenic poplar led to a reduction in lignin content (up to 45%) and an increased growth rate. These researchers also claimed a 9–15% increase in cellulose content and some alteration of hemicellulose composition in their transgenic lines. These latter claims are open to interpretation, however, because of the small statistical sample and the authors' decision to report lignin and cellulose contents as percentages of dry wood weight without also providing total biomass yields. It will be interesting to see whether manipulation of the genes encoding other 4-coumarate:CoA ligase isozymes might provide a further avenue for adjusting lignin composition, as was demonstrated for *Arabidopsis* [40].

Environmental considerations

As noted by Mullin and Bertrand [41], the economic equations that drive investment into the production of improved forest trees do not always support efforts to modify wood quality traits, such as lignin extractability, because returns on that investment cannot be realized prior to harvest of the improved wood. By contrast, trees enhanced with genes that provide for gains in growth and yield are seen to impact profits immediately by enabling faster rotations. Unfortunately, transgenes for pest and disease resistance, as well as those for herbicide and stress tolerance, by their nature improve host fitness, and thus carry the potential for more far-reaching effects should they be passed to subsequent generations of feral trees. Activist groups with agendas that include prohibition of environmental release of genetically engineered trees and crops have worked hard to make 1999 a watershed year for public opinion, and transgenic trees having increased fitness have been a prime target for their criticism. The research community ignores public debate on

this topic at its own peril. James *et al.* [42] have described potential impacts from escape of such trees, outlined some potential avenues to decrease the possibility for such escapes, and have encouraged increased field testing and monitoring. Many of their suggestions were incorporated into a position statement on the benefits and risks of transgenic tree plantations adopted by the IUFRO Working Party on Molecular Biology of Forest Trees (2.04.06) in September 1999 [43]. Copies of this statement may be obtained via the Internet (http://www.fsl.orst.edu/tgerc/iufro_pos-statm.htm).

Conclusions

Forest tree improvement has heretofore been a slow and arduous process by virtue of the large size and long generation times of trees. Biotechnology has now provided tools, however, that allow us to select and engineer superior trees with much the same speed and efficiency that can be applied to other organisms. Consequently, the new millennium is likely to see large and rapid changes in the woody species used to manufacture paper and solid wood products. Major changes in lignin composition and content have already been achieved through genetic engineering and the use of marker-aided selection, and trees with enhanced fitness traits are ready for field testing. However, the impact that such trees may have on ecosystems when planted on the plantation scale needs much additional study.

Update

Recent results have shown that diversion of hydroxycinnamates into either the lignin biosynthetic pathway or pathways leading to other secondary metabolites is partially controlled through complex feedback mechanisms at the enzymatic level [44]. Thus, levels of coniferyl and 5-hydroxyconiferyl aldehydes, intermediates in the latter steps of the lignin biosynthetic pathway, may govern the activity of the enzymes necessary for sinapyl alcohol production.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Merkle SA, Trigiano RN: **In vitro propagation of hardwoods.** In *Applications of Vegetative Propagation in Forestry; Proceedings of the 1992 SRIEG Biennial Symposium on Forest Genetics: 1992 July 8–10; Huntsville, AL.* Edited by Foster GS, Diner AM. New Orleans, LA: USDA Forest Service General Technical Report SO-108: Southern Forest Experiment Station; 1992:23-37.
2. Li XY, Huang FH, Gbur EE: **Polyethylene glycol-promoted development of somatic embryos of loblolly pine (*Pinus taeda* L.).** *In Vitro Cell Dev Biol Plant* 1997, **33**:184-189.
3. Li XY, Huang FH, Gbur EE: **Effect of basal medium, growth regulators and Phytigel concentration on initiation of embryogenic cultures from immature zygotic embryos of loblolly pine (*Pinus taeda* L.).** *Plant Cell Rep* 1998, **17**:298-301.
4. Li XY, Huang FH, Murphy JB, Gbur EE: **Polyethylene glycol and maltose enhance somatic embryo maturation in loblolly pine (*Pinus taeda* L.).** *In Vitro Cell Dev Biol Plant* 1998, **34**:22-26.
5. Klimaszewska K, Smith DR: **Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum.** *Physiol Plant* 1997, **100**:949-957.
6. Lelu MA, Bastien C, Drugeault A, Gouez ML, Klimaszewska K: **Somatic embryogenesis and plantlet development in *Pinus sylvestris* and *Pinus pinaster* on medium with and without growth regulators.** *Physiol Plant* 1999, **105**:719-728.
This study shows that for *P. sylvestris* (and potentially for other conifers), all steps of embryogenesis, from induction to maturation, can occur without any exposure to plant growth regulators, and that manipulation of gellan gum concentration can improve pine somatic embryo maturation.
7. Cairney J, Xu NF, Pullman GS, Ciavatta VT, Johns B: **Natural and somatic embryo development in loblolly pine – gene expression studies using differential display and DNA arrays.** *Appl Biochem Biotechnol* 1999, **77-9**:5-17.
Over 400 ESTs identified by differential display as having altered expression patterns during loblolly pine zygotic embryo development were used to produce macroarrays. These macroarrays were used to monitor the expression of genes during somatic embryo development under varied culture conditions to look for conditions that would more closely mirror the gene expression patterns in developing zygotic embryos.
8. Smith DR: **Successful rejuvenation of radiata pine.** In *Proceedings of the 25th Biennial Southern Forest Tree Improvement Conference: 1999 July 11–14; New Orleans, LA.* New Orleans, LA: Southern Forest Tree Improvement Committee; 2000: in press.
This study is the first report of induction of somatic embryogenesis from tissues (apical meristems) of mature pine trees, and provides an excellent example of the economics of applying such an approach for selectively propagating mature trees based on their individual fiber properties.
9. Merkle SA, Battle PJ: **Enhancement of embryogenic culture initiation from tissues of mature sweetgum trees.** *Plant Cell Rep* 2000, **19**:268-273.
10. Bandyopadhyay S, Cane K, Rasmussen G, Hamill JD: **Efficient plant regeneration from seedling explants of two commercially important temperate eucalypt species – *Eucalyptus nitens* and *E. globulus*.** *Plant Sci* 1999, **140**:189-198.
11. Cid LPB, Machado ACMG, Carnevali SBRC, Brasileiro ACM: **Plant regeneration from seedling explants of *Eucalyptus grandis* × *E. urophylla*.** *Plant Cell Tiss Org Cult* 1999, **56**:17-23.
12. Tremblay L, Levasseur C, Tremblay FM: **Frequency of somaclonal variation in black spruce (*Picea mariana*, Pinaceae) and white spruce (*P. glauca*, Pinaceae) derived from somatic embryogenesis and identification of some factors involved in genetic instability.** *Am J Bot* 1999, **86**:1373-1381.
13. Park YS, Barrett JD, Bonga JM: **Application of somatic embryogenesis in high-value clonal forestry: development, genetic control, and stability of cryopreserved clones.** *In Vitro Cell Dev Biol Plant* 1998, **34**:231-239.
14. DeVerno LL, Park YS, Bonga JM, Barrett JD: **Somaclonal variation in cryopreserved embryogenic clones of white spruce [*Picea glauca* (Moench) Voss.].** *Plant Cell Rep* 1999, **18**:948-953.
15. Walter C, Grace LJ, Wagner A, White DWR, Walden AR, Donaldson SS, Hinton H, Gardner RC, Smith DR: **Stable transformation and regeneration of transgenic plants of *Pinus radiata* D.** *Don Plant Cell Rep* 1998, **17**:460-468.
The first published report of stable genetic transformation and regeneration of any pine. Microprojectile bombardment of embryogenic cultures with constructs carrying NPTII and GUS, followed by selection on geneticin was used to obtain transformed embryogenic material from which transgenic somatic seedlings were regenerated.
16. Charest PJ, Devantier Y, Lachance D: **Stable genetic-transformation of *Picea mariana* (black spruce) via particle bombardment.** *In Vitro Cell Dev Biol Plant* 1996, **32**:91-99.
17. Klimaszewska K, Devantier Y, Lachance D, Lelu MA, Charest PJ: ***Larix laricina* (tamarack) – somatic embryogenesis and genetic transformation.** *Can J Forest Res* 1997, **27**:538-550.
18. Tian LN, Levee V, Mentag R, Charest PJ, Seguin A: **Green fluorescent protein as a tool for monitoring transgene expression in forest tree species.** *Tree Physiol* 1999, **19**:541-546.
19. Wenck AR, Quinn M, Whetten RW, Pullman G, Sederoff R: **High efficiency *Agrobacterium*-mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*).** *Plant Mol Biol* 1999, **39**:407-416.
Evidence is presented that manipulation of vir genes can enhance *Agrobacterium*-mediated transformation of conifers, such as Norway spruce and loblolly pine embryogenic cultures.
20. Levee V, Garin E, Klimaszewska K, Seguin A: **Stable genetic transformation of white pine (*Pinus strobus* L.) after cocultivation**

- of embryogenic tissues with *Agrobacterium tumefaciens*. *Mol Breed* 1999, 5:429-440.
21. Han KH, Meilan R, Ma C, Strauss SH: **An *Agrobacterium* transformation protocol effective on a variety of cottonwood hybrids (genus *Populus*)**. *Plant Cell Rep* 2000, 19:315-320.
 22. Ho CK, Chang SH, Tsay JY, Tsai CJ, Chiang VL, Chen ZZ: ***Agrobacterium tumefaciens*-mediated transformation of *Eucalyptus camaldulensis* and production of transgenic plants**. *Plant Cell Rep* 1998, 17:675-680.
 23. Rugh CL, Senecoff JF, Meagher RB, Merkle SA: **Development of transgenic yellow poplar for mercury phytoremediation**. *Nat Biotechnol* 1998, 10:925-928.
- Yellow-poplar trees engineered with a modified bacterial mercuric ion reductase gene via microprojectile bombardment of embryogenic cultures were capable of surviving on medium with up to 50 μ M ionic mercury by reducing it to less toxic metallic mercury.
24. Allona I, Quinn M, Shoop E, Swope K, St Cyr S, Carlis J, Riedl J, Retzel E, Campbell MM, Sederoff R, Whetten RW: **Analysis of xylem formation in pine by cDNA sequencing**. *Proc Natl Acad Sci USA* 1998, 95:9693-9698.
 25. Sterky F, Regan S, Karlsson J, Hertzberg M, Rohde A, Holmberg A, Amini B, Bhalerao R, Larsson M, Villarreal R *et al.*: **Gene discovery in the wood-forming tissues of poplar: analysis of 5,692 expressed sequence tags**. *Proc Natl Acad Sci USA* 1998, 95:13330-13335.
 26. Devey ME, Sewell MM, Uren TL, Neale DB: **Comparative mapping in loblolly and radiata pine using RFLP and microsatellite markers**. *Theor Appl Genet* 1999, 99:656-662.
 27. Tuskan G, West D, Bradshaw HD, Neale D, Sewell M, Wheeler N, Megraw B, Jech K, Wiseloge A, Evans R *et al.*: **Two high-throughput techniques for determining wood properties as part of a molecular genetics analysis of hybrid poplar and loblolly pine**. *Appl Biochem Biotechnol* 1999, 77-79:55-65.
- Computer tomography X-ray densitometry and pyrolysis molecular beam mass spectrometry were used to analyze the specific gravity and composition of wood from 375 hybrid poplars and 172 loblolly pines, each of which represented segregating progeny from a three-generation pedigree. Eleven quantitative trait loci linked to specific wood components and two quantitative trait loci linked to multiple components were mapped in loblolly pine.
28. Sederoff RR, MacKay JJ, Ralph J, Hatfield RD: **Unexpected variation in lignin**. *Curr Opin Plant Biol* 1999, 2:145-152.
 29. Baucher M, Monties B, Van Montagu M, Boerjan W: **Biosynthesis and genetic engineering of lignin**. *Crit Rev Plant Sci* 1998, 17:125-197.
 30. Halpin C, Holt K, Chojecki J, Oliver D, Chabbert B, Monties B, Edwards K, Barakate A, Foxon GA: **Brown-midrib maize (bm1) – a mutation affecting the cinnamyl alcohol dehydrogenase gene**. *Plant J* 1998, 14:545-553.
 31. MacKay JJ, O'Malley DM, Presnell T, Booker FL, Campbell MM, Whetten RW, Sederoff RR: **Inheritance, gene expression, and lignin characterization in a mutant pine deficient in cinnamyl alcohol dehydrogenase**. *Proc Natl Acad Sci USA* 1997, 94:8255-8260.
 32. Ralph J, MacKay JJ, Hatfield RD, O'Malley DM, Whetten RW, Sederoff RR: **Abnormal lignin in a loblolly pine mutant**. *Science* 1997, 277:235-239.
 33. MacKay J, Presnell T, Jameel H, Taneda H, O'Malley D, Sederoff R: **Modified lignin and delignification with a CAD-deficient loblolly pine**. *Holzforchung* 1999, 53:403-410.
 34. Lapiere C, Pollet B, Petit-Conil M, Toval G, Romero J, Pilate G, Leple JC, Boerjan W, Ferret V, De Nadai V, Jouanin L: **Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid O-methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping**. *Plant Physiol* 1999, 119:153-163.
 35. Zhong RQ, Morrison WH, Negrel J, Ye ZH: **Dual methylation pathways in lignin biosynthesis**. *Plant Cell* 1998, 10:2033-2045.
 36. Tsai CJ, Popko JL, Mielke MR, Hu WJ, Podila GK, Chiang VL: **Suppression of O-methyltransferase gene by homologous sense transgene in quaking aspen causes red-brown wood phenotypes**. *Plant Physiol* 1998, 117:101-112.
 37. Humphreys JM, Hemm MR, Chapple C: **New routes for lignin biosynthesis defined by biochemical characterization of recombinant ferulate 5-hydroxylase, a multifunctional cytochrome P450-dependent monooxygenase**. *Proc Natl Acad Sci USA* 1999, 96:10045-10050.
 38. Osakabe K, Tsao CC, Li LG, Popko JL, Umezawa T, Carraway DT, Smeltzer RH, Joshi CP, Chiang VL: **Coniferyl aldehyde 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms**. *Proc Natl Acad Sci USA* 1999, 96:8955-8960.
 39. Hu WJ, Harding SA, Lung J, Popko JL, Ralph J, Stokke DD, Tsai CJ, Chiang VL: **Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees**. *Nat Biotechnol* 1999, 17:808-812.
- Transgenic aspen in which xylem-specific 4-coumarate:coenzyme A ligase (4CL) was repressed by an antisense gene had a 45% reduction in lignin content. The trees also had thicker stems, longer internodes, larger leaves, and their cuttings showed better rooting than controls.
40. Lee D, Meyer K, Chapple C, Douglas CJ: **Antisense suppression of 4-coumarate:coenzyme A ligase activity in *Arabidopsis* leads to altered lignin subunit composition**. *Plant Cell* 1997, 9:1985-1998.
 41. Mullin TJ, Bertrand S: **Environmental release of transgenic trees in Canada – potential benefits and assessment of biosafety**. *Forestry Chron* 1998, 74:203-219.
 42. James RR, DiFazio SP, Brunner AM, Strauss SH: **Environmental effects of genetically engineered woody biomass crops**. *Biomass Bioener* 1998, 14:403-414.
 43. Strauss S, Boerjan W, Cairney J, Campbell M, Dean J, Ellis D, Jouanin L, Sundberg B: **Forest biotechnology makes its position known**. *Nat Biotechnol* 1999, 17:1145.
 44. Li L, Popko JL, Umezawa T, Chiang VL: **5-hydroxyconiferyl aldehyde modulates enzymatic methylation for syringyl monolignol formation, a new view of monolignol biosynthesis in angiosperms**. *J Biol Chem* 2000, 275:6537-6545.