

# Applying biotechnology to design tree composition for value-added products: a mini-review

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## Summary

A major goal for forest biotechnology is the modulation of tree phenotypes for industrial applications. Such modulation is based on understanding the relationship between genotype and phenotype. Further, the capacity to control gene regulation and expression in a highly targeted manner is a critical component in new methods for achieving this targeted modulation. As such, biotechnology is vital to the continued improvement of existing forest products and the development of aspects of a viable bioeconomy. Such a bioeconomy will be based on differentiated value-added crops and animal breeds for food, feed and health. In a forestry context, novel uses of trees will potentially include traditional and advanced biofibre applications, bioremediation and products from biorefineries: for example, biodegradable plastics and feedstocks. To date biorefinery concepts have emphasised the production of lignin and polyphenolics that have considerable potential for the manufacture of high-value products. This paper discusses such developments and assesses the potential for biotechnology to address these complex questions.

**Keywords:** biotechnology; forest products; chemicals; fuels; fibres; plastics; tree breeding

## Introduction

Australia's forest and wood products industries have an annual gross turnover of more than A\$18 billion, directly or indirectly employ over 130 000 people, and contribute about 1% to Australia's GDP (Bureau of Rural Sciences 2005). The industries continue to attract new investment into resources and processing from domestic and foreign companies:

- During 2000–2005, more than A\$2.2 billion were invested in wood fibre processing facilities.
- Potentially, a further A\$2.5–3 billion is likely to be invested over the next few years, including two new pulp and paper mills that would go a long way towards addressing Australia's balance of trade deficit in forest products.

The growth in Asian consumption of forest products, increasing domestic consumption and increasing wood supply in Australia is creating an environment for investment in new processing capac-

ity in Australia, and the industry has an opportunity to transition to a diversified forest biorefinery-based future. Implementing this will involve the development of new manufacturing processes and the manufacture of novel bioproducts that will potentially have new supply chains, raw material quality parameters, investors and end-users.

The ability of trees, in particular fast-growing species native to Australia such as *Eucalyptus*, to accumulate large amounts of biomass is directly related to secondary xylem growth and development. The morphology and chemistry of the secondary xylem are the key determinants of wood quality for structural uses, pulp and paper, carbon capture and cell wall deconstructability (i.e. for biofuels applications). For the Australian forest industry, and indeed the global industry, success in future world bioeconomy markets will require forest management and improvement strategies that are based on a fundamental understanding of the biochemical pathways determining cell wall morphology and chemistry, and the effects that silvicultural regimes and environmental change can have on them. Elucidating the genetics, genomics and metabolomics of different trees exhibiting desirable macro-phenotypes, and calibrating field technologies (such as near-infrared spectroscopy (NIRS)) with them will be a method for directing forest management and improvement schemes in real time and will provide insights into the biochemistry underpinning wood formation. Mirroring genomics and phenomics techniques developed for human health applications, such a program will provide a suite of functional post-genomics methods for tying together basic gene discovery work and advanced detection of sub-cellular biochemical variation (e.g. field NIRS calibrated for metabolite detection via liquid chromatography–mass spectrometry (LC–MS), nuclear magnetic resonance spectroscopy (NMRS), etc.) to provide a non-destructive diagnostic tool to predict standing tree performance.

## Issues and opportunities: complexity of the lignocellulosic resource

Cellulose is a biopolymer that is renewable, super-abundant and whose potential for conversion into economically viable cellulosic ethanol biofuels has been well documented (Hahn-Hägerdahl

*et al.* 2006; Rubin 2008; US DOE Bioenergy Research Centers 2009). In plant fibres,  $\beta$  1-4 linked cellulose affords the mechanical properties of the crystalline cellulose which is arranged in microfibrils enclosed by hemicelluloses and lignin. As well as these microfibrils, there exist nanofibres (also composed by cellulose) with diameters of 5–50 nm and lengths of several millimetres conformed by nanocrystalline domains and amorphous regions. The amorphous regions act as structural defects and are responsible for the transverse cleavage of the microfibrils into short monocrystals under acid hydrolysis. These nanocellulose crystals make up to 20% by mass of wood and have found some applications as reinforcements in composite materials, as well as displaying interesting liquid crystal properties (nematic and chiral nematic). The mechanical properties of nanocellulose crystals, such as a tensile strength twice that of steel wire but with a comparable modulus, a tensile strength 25% and a modulus 25–50% of carbon nanotubes but at a small fraction of the cost (Beecher 2007), make them extremely effective as reinforcing agents in polymeric materials with the potential to create a green bio-steel material. The accessible anisotropic surface chemistry of the crystals allows for ready chemical modification. The crystals are biologically compatible and could be used in areas non-traditional to the forest industry such as scaffolding for medical applications and reinforcement for shape memory polymers (Auad *et al.* 2008). The major impediment to their rapid application in a range of industries is the current gap in the ability to scale up the production of these materials from the lab and the lack of knowledge of how the manufacturing process can affect the fundamental properties of these materials.

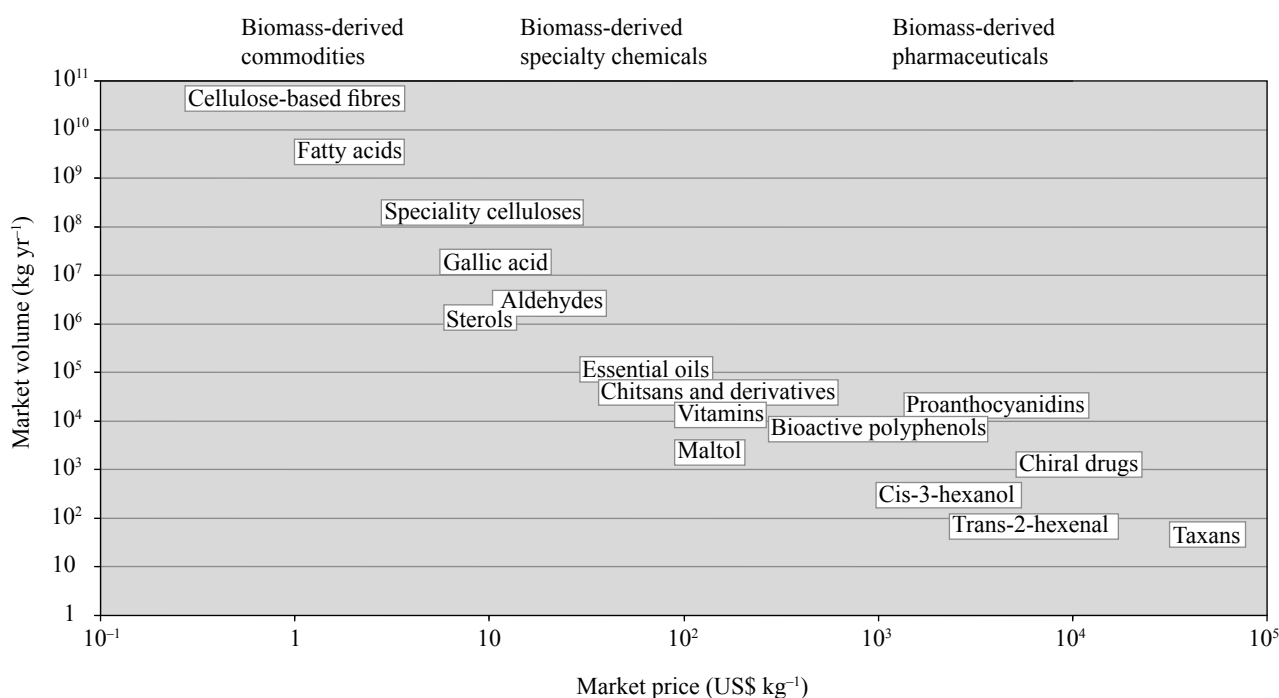
In parallel with the potential for cellulose-based products, current biorefinery concepts place particular emphasis on lignin and polyphenolics that have considerable potential for the manufacture of high-value products (Towers *et al.* 2007; Chambost and Stuart

2009). Lignin is the world's most abundant aromatic polymer and yet, despite this inherent chemical potential, currently in pulp and paper mills 98–99% of kraft/sulfite lignins are used merely as fuel for process chemical recovery. Only 1–2% of these lignins are used for specialty chemicals such as dispersants, emulsifiers and binders. The reasons for this untapped potential are multifarious, but in general conversion of lignin to high-value products is hindered by its biochemical complexity, heterogeneity, polydispersity and high levels of impurities (Boudet *et al.* 2003). Nevertheless, there is a major opportunity to move the traditional forest and forest products industry from commodity to value-added products based on cellulose and, primarily, lignin chemistry. Some potential product lines are illustrated in Figure 1. Whilst much potential for improvement and diversification exists in processing and feedstock optimisation via traditional breeding programs, biotech solutions also hold considerable promise to assist rapid assessment of wood chemistry by applying biomarkers, gene-assisted selection (GAS) programs for tree improvement and ultimately the modification of the lignified cell wall via genetic manipulation or via microbial systems.

To take one example, aromatic aldehydes, ketones and acids can be efficiently synthesised from acid-soluble lignin by alkaline oxidation with  $\text{Fe}^{3+}$  and  $\text{Cu}^{2+}$  as catalysts, and these can be further converted to valuable materials such as PDC (2*H*-pyran-2-one-4,6-dicarboxylic acid), a monomer for polyester and polyamide plastic synthesis (Shigehara *et al.* 2001; Xiang and Lee 2002).

Other potential products of the forest biorefinery (Fig. 2) include:

- Combustion
  - energy
- Complete degradation (pyrolysis)
  - methane, CO, syngas



**Figure 1.** Market size and price for forest-biomass-derived value-added products

- Partial degradation
  - phenolics—synthesis of polymers and resins
- Hybrid adhesive systems (phenolics + oils + tannin adhesives) for fibre composite systems
- Papermaking additive replacement (e.g. binder systems for coated papers)
- Sulphur-free lignin:
  - produced during bioethanol production, solvent or soda pulping
  - superior properties
  - substitute for phenolic powder resins (e.g. brake pads, OSB binders)
  - polyurethane foams
  - epoxy resins.

## Wood extractives

Wood extractives are non-cell wall components that can be removed using solvents, for example petroleum ether, acetone, ethanol or water. They are relatively small molecules (<C40) and usually comprise 1–5% of the wood. Their composition is known to be under varying levels of genetic control (Yanchuk *et al.* 1988; Fries *et al.* 2000):

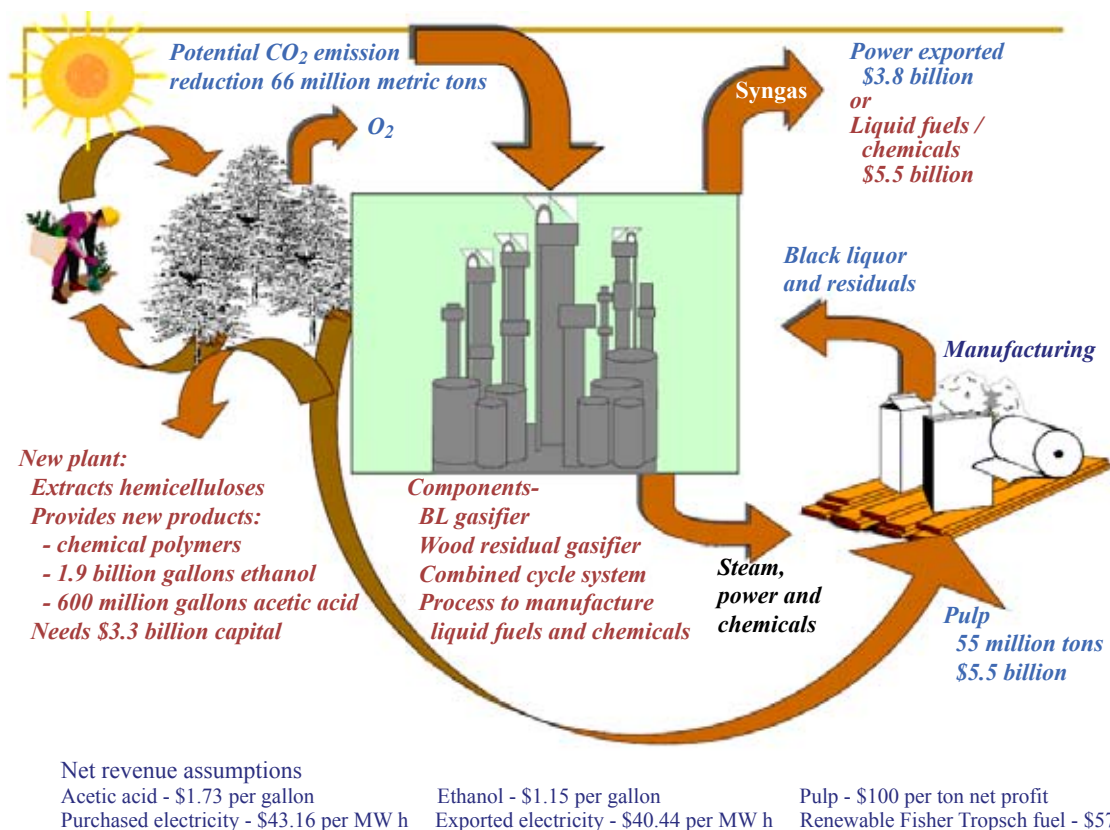
- Softwood:
  - resin acids: 40–45% of extractives
  - fatty acids: 40–60%
  - monoterpenes (turpentine): 0.2–0.8%
  - phenolics: 0.03%.

- Hardwood:
  - fatty acids: 60–90%
  - phenolics: 0.03%.

Wood extractives can be routinely fingerprinted using gas chromatography–mass spectrometry (GC–MS) technology (Fernandez *et al.* 2001) and are known to have many potential uses in the bioeconomy. Table 1 delineates a number of such uses.

**Table 1.** Some wood extractive categories and their potential uses

Extractive	Utility
Fatty acids	
Linoleic acid	Dietary
Suberin	Polyester
$\beta$ -sitosterol	Dietary (functional food additive)
Terpenoids	
Monoterpenes (pinene, limonene)	Fragrances, flavours
Diterpenes (abietic, pimaric acids)	Resins, sizing agents
Triterpenes (betulin)	Medicinal
Phenolics	
Stilbenes (pinosylvin)	Bioactive phenolics
Flavonoids	Anti-oxidants
Lignans	Viral, anti-cancer treatments



**Figure 2.** The forest biorefinery concept. The figures are for the USA annually ('Agenda 2020' program) (adapted from a figure provided by courtesy and Ben Thorp of Flambeau River BioRefinery, Wisconsin, USA, and Shri Ramaswamy, University of Minnesota, USA, 2009).

An excellent example of the utility of wood extractives, produced either as by-products of traditional processes or as co-product classes, is provided by betulin—a bioactive phenolic compound with medicinal applications (Jager *et al.* 2008). Betulin, which can be extracted from the outer bark of such commercial species as *Eucalyptus globulus*, has been shown to help wounds heal faster and reduce inflammation. Many cosmetic companies, touting it as a skin toner and restorer, add birch bark extract to various products. Betulin can be easily converted to betulinic acid, which possesses a wide spectrum of biological and pharmacological properties such as anti-malarial and anti-inflammatory activities, anti-HIV activity and cytotoxicity against a variety of tumour cell lines comparable to some clinically used drugs. Betulinic acid is specifically cytotoxic to several tumour cell lines (melanomas) by inducing apoptosis in cells.

### Options for progress—biotechnology

Biotechnology now offers the possibility of designing, or at least selecting for, secondary-cell-wall-derived biomass with properties dictated by, and tailored for, given downstream applications of the types delineated above. The major routes for accessing this possibility are demonstrated via advanced breeding/clonal forestry/propagation/rejuvenation technologies, biotech-enabled monitoring of resource or biotech for manipulation of resource. Examples of each of these approaches form the remainder of this paper.

#### Advanced breeding

Traditional tree breeding programs are, by their very nature, long-term, multi-generational projects. They involve the collection of breeding values and heritabilities for multiple traits on a large scale. Whilst remarkable progress can be made for simple traits, such as oil production (Butcher *et al.* 1996), in order to dissect the genetic control of wood formation and the genetic relationship among wood traits from genomics and quantitative genetics, many problematic technical issues must be overcome. One such initiative is the CSIRO-led Juvenile Wood Initiative project which determined that large and significant genotype  $\times$  site (or silviculture) interactions occur on juvenile–mature wood transition in radiata pine trees and that significant adverse genetic correlations exist among growth and wood traits (Baltunis *et al.* 2007; Matheson *et al.* 2008). Further work is currently planned to address these issues. However, as mentioned, there do exist examples of value-added gain in forest production from application of traditional quantitative genetics methods. Tea tree (*Melaleuca*) oil production is a comparatively small but significant industry in Australia (RIRDC 2006). The oil is a value-added product with applications in personal care, household, agricultural and veterinary areas. Recently, a 9-year program of traditional genetic improvement was completed and resulted in an 80% genetic gain in oil production (Doran *et al.* 2006). It could be envisaged that a similar approach to biodiesel production could yield similar results.

### Monitoring the resource

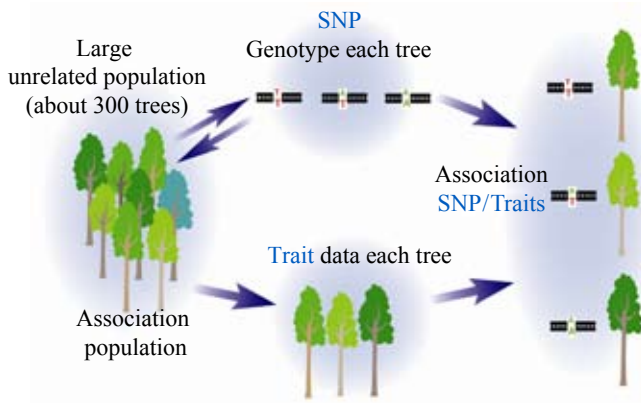
#### Association genetics and gene-assisted selection

In agricultural biotechnology, molecular breeding can be broken down into two approaches—transformation technologies (genetic modification or GM) and molecular-marker-assisted selection (MAS) technologies (Potter 2005; Gonzalez-Martinez *et al.* 2007, 2008; Ingvarsson *et al.* 2008; Thumma *et al.* 2009). The potential of MAS for forest tree improvement has increased dramatically in recent years because of significant advances in genomics technology. Unlike agricultural crop breeding, forest tree breeding is still in its infancy and conventional breeding has the potential to make large gains in tree improvement programs. MAS—or more specifically gene-assisted selection (GAS)—can deliver improvements without the complications inherent in GM from a biosafety and bioethics perspective. One promising approach to MAS is that it should complement and enhance conventional tree breeding programs, providing advantages that:

- enhance conventional breeding by making it more targeted and accurate
- accelerate product development cycles by enabling selection at the seedling stage
- allow work with traits that are difficult or prohibitively expensive to measure.

Research programs in wood quality and genetics have made important advances in our understanding of the genetic control of wood traits in eucalypts. Population-based ‘association studies’ (Fig. 3) are poised to deliver major advances in forest genetics in the next decade. Pioneering this approach, researchers have now used association studies to link specific alleles in two eucalypt genes with the important wood traits of microfibril angle (MFA) (Thumma *et al.* 2005) and pulp yield (Thumma *et al.* 2009). Associations have also been published in three conifer species to date (Gonzalez-Martinez *et al.* 2007, 2008; Ingvarsson *et al.* 2008; Eckert *et al.* 2009).

Recent progress in wood quality and genetics has led to the development of genetic linkage maps and the discovery of quantitative trait loci (QTL) for fibre length and other traits in both *E. nitens* and *E. globulus* (Byrne *et al.* 1997; Thamarus *et al.* 2004) and in poplars (Potter and Watson 2004). Family-based QTL analysis studies have been valuable in gaining insights into the genetic basis of control of wood traits. However, application of these results in tree improvement programs has been limited. Some of the reasons behind the low impact of QTL studies include the low resolution of marker-trait associations due to extended linkage disequilibrium (LD) in pedigrees (i.e. markers are often millions of base pairs away from the causative DNA variant); the low proportion of phenotypic variation explained by individual QTLs (generally less than 10% of total genetic variance); and the results from such studies are frequently limited to the particular family in which the QTLs are discovered.



**Figure 3.** A schematic of an association genetics experiment

The emerging field of association genetics offers the potential to circumvent most of the problems encountered with QTL studies (Sewel and Neale 2000). CSIRO research shows that it is possible to detect marker–trait associations at high resolution by taking advantage of low LD in natural populations (i.e. small regions within a gene associated with a trait can be identified). This has led to publication of the first association study in a forest tree species (Thumma *et al.* 2005). Genomics research is revealing a large number of genes that are likely to be involved in cell wall development and thus affect fibre properties such as MFA and pulp yield (Thumma *et al.* 2009). Gene-based association studies are particularly attractive in eucalypts because of the recent release of a publicly available complete genome sequence (the final assembly phase is now in progress courtesy of the US Department of Energy Joint Genome Institute (JGI) and the relatively small genome size and low duplication of the genome compared to that of conifers. Over 150 candidate genes that may influence pulp yield and other wood quality traits such as cellulose, lignin, density and MFA have been identified, and completed association studies exist in over 20 eucalypt genes (Thumma *et al.* 2005, 2009). Such research successes to date strongly suggest that association studies will yield many more valuable DNA markers that can be immediately used for gene-assisted selection for accelerated and highly targeted forest tree improvement.

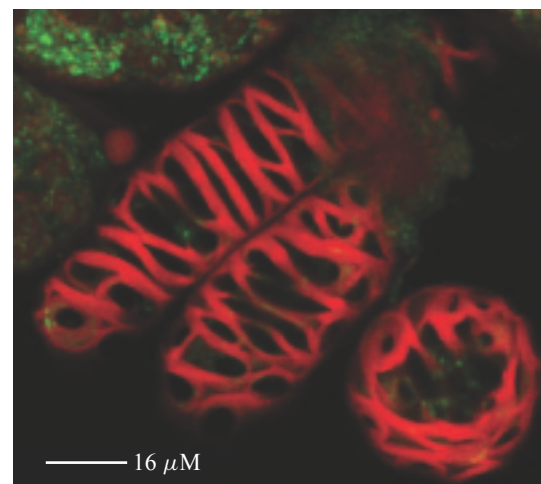
### Metabolic profiling

Metabolic profiling (metabolomics) is one of the newest and most promising technologies in the area of functional genomics (Dunn and Ellis 2005). This technology facilitates an understanding of in vivo sub-cellular level biochemical interactions, by direct elucidation of product expression. Metabolic profiling provides insight into the ultimate functions of gene expression and is a key to understanding how changes at the genome (and proteome) level affect cellular function (Bino *et al.* 2004). Metabolites are the low-molecular-weight molecules that arise as intermediates in the expression of an organism's phenotype—in parallel to the term 'genome' which describes the complete set of genes, the complete set of small molecules in a tissue of an organism is known as the 'metabolome'. These small molecules fulfil a variety of roles: some participate in cellular signalling or energy and nutrient cycling, while others are precursors for macromolecules, structural polymers and defence-related biomaterials. The

regulation of metabolism is the final point for the organism to adjust in response to environmental stimuli. This control is achieved by spatial and temporal organisation of structural components, enzymes and substrates within the cell, all downstream from the control points for protein expression. Thus, the output of a specific set of small molecules during cellular activity represents the ultimate response of biological systems to genetic, stress or environmental change. The small molecules generated during such cellular activity may be regarded as the ultimate gene products, and their relative and absolute abundance will provide a robust indication of phenotype (Dixon *et al.* 2006). Small secondary metabolites, which ultimately represent the catabolic and anabolic activities being performed at any given time, facilitate the generations of 'biochemical snapshots' that represent a trait in formation or response signature created. Changes in the genotype and its interaction with the environment can therefore be manifested through immediately observable changes in the metabolome. Metabolic profiling has proven to be a very sensitive means to characterise the genetic and or environmental factors that affect biochemical traits in crop plants, and are likely to be similarly applicable to tree species (Seger and Sturm 2006). Metabolic profiling thus provides an innovative tool for decoding the biochemical functions of plant genes and identifying the regulatory networks operating in plant metabolism (Oliver *et al.* 1998; Kitano 2002; Lodish *et al.* 2004). Elucidating the metabolic profiles of different trees exhibiting specific phenotypes could potentially be a means of characterising and selecting clonal lines of desirable trees for use in plantations. Equally important, this approach will provide novel insights related to the biochemistry of wood formation and forest health.

### Genetic manipulation

In recent years, numerous transgenic plants have been generated with altered expression of one or more genes involved in lignification (Anterola *et al.* 2002; Boerjan *et al.* 2003; Boudet *et al.* 2003). In general, these plants with modified lignin biosynthesis have been produced with the aim of improving plant raw materials for pulp and paper production. Such genetic



**Figure 4.** Modified tracheary elements via genetic manipulation of the *HCT* gene (from Wagner *et al.* 2007)

engineering has yielded new insights into how the lignin biosynthetic pathway operates and demonstrates that lignin can be improved to facilitate bioproducts extraction. Importantly, fine-tuned modification of lignin content, composition, or both is now achievable and could have important economic and environmental benefits. The work of Wagner *et al.* into metabolic plasticity and *HCT*-gene silencing in softwoods (Fig. 4; Wagner *et al.* 2007) is an excellent demonstration of this concept. Their work using an RNAi-mediated tracheary element model system showed that modification of the *HCT* gene had strong effects on lignin content, monolignol composition and inter-unit linkages, providing an important metabolic entry point for modified methoxylated phenylpropanoid synthesis.

## Conclusions—transforming forestry

Traditionally, added value from biology has come from the targeted control of phenotype. The capacity to control gene regulation and expression via modern biotechnology in a highly targeted manner is a vital component in the new methods for achieving this. In a forestry context, control of tree phenotype via such precise methods has and will continue to lead to novel uses of trees in the bioeconomy including biofibre applications, carbohydrates, ligno-cellulosic biofuels, bioremediation, biodegradable plastics, oils, industrial feedstocks and biopharma products. Already, trees are increasingly being used as factories to make new advanced materials—in a global market, opportunities stretch from 5 t y<sup>-1</sup> (@\$100 000 kg<sup>-1</sup>—taxans) to 100 billion t y<sup>-1</sup> (@\$1 kg<sup>-1</sup>—bio-functionalised cellulose fibres for advanced composites: Jandura *et al.* 2001). Particular emphasis has been placed on lignin and polyphenolics that have considerable potential for the manufacture of high-value products. Biotech modification of lignin can produce useful thermoplastics. Forest-derived bioactive polyphenolics can be applied to health protection (e.g. anti-oxidant properties) and disease treatment (viral and cancer treatments with podophyllotoxin/nor-dihydroguaiaretic acid derivatives). Lignification can be extensively modified through biotechnology, for example via upregulated or silenced biosynthetic pathway genes. However, the phenotype of the resultant plants is not always predictable because of the complex nature of the biosynthetic pathway for lignification. The process can be assisted and directed by reverse engineering desirable phenotypes using techniques such as association genetics and metabolomics. By these methods, forestry will be transformed from a traditional, commodities-based industry to a value-added industry enabled by early screening of populations for superior alleles, much shorter breeding cycles and highly selective choices based on downstream applications, leading to trees with new capacities.

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