Lignin Studies of Transgenic Norway Spruce

Johan Wadenbäck
Faculty of Natural Resources and Agricultural Sciences
Department of Plant Biology and Forest Genetics
Uppsala

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2006

Acta Universitatis Agriculturae Sueciae
2006: 64

Front cover illustration:
The most common linkage type in Norway spruce connecting monolignol units in lignin; the arylglycerol β-aryl ether structure (β-O-4 linkage) between a guaiacyl- (G) (left) and a *p*-hydroxyphenyl (H) unit (right).

ISSN 1652-6880
ISBN 91-576-7113-3

Tryck: SLU Service/Repro, Uppsala 2006

Abstract

Wadenbäck, J. 2006. *Lignin studies of transgenic Norway spruce*. Doctor's dissertation.
ISSN 1652-6880, ISBN 91-576-7113-3.

An attractive objective in tree breeding is to reduce the content of lignin, or increase its extractability, to favor delignification during pulping. This study investigates the potential for lignin alterations in Norway spruce (*Picea abies* [L.] Karst) using genetic engineering. Young plants from seven lines transformed with an antisense construct of a gene encoding cinnamoyl CoA reductase (CCR), an important enzyme of lignin biosynthesis, have been compared with control plants. Transgene copy-number varied from 1-8 and the transcript abundance of *CCR* was reduced by up to 50%. The transcript abundances of lignin biosynthetic genes were positively correlated, especially for those at the end of the pathway. The lignin content and the non-condensed *p*-hydroxyphenyl (H) lignin fraction was reduced 5% and 23%, respectively. No increase in delignification from pulps from the most down-regulated lines was observed, suggesting an increased proportion of condensed bonds in the lignin.

Lignin characters were compared in one-year-old plants and nine-year-old trees of full-sib families. The lignin did not vary within or among the families at the same age. However, the lignin content and the non-condensed H-lignin fraction were increased 15% and 75%, respectively, in the trees, indicating compression wood. In addition, the concentration of C9-units and the ratio of *erythro*- and *threo* stereoisomers varied among the families with age.

Possibilities for early selection among *asCCR* embryogenic cultures were evaluated by microarray analysis. Down-regulation of *CCR* does not affect the transcript abundance of genes regulating lignin biosynthesis. However, several genes in pathways associated with lignin biosynthesis were affected. Functional assignment by Gene Ontology terms showed that transformation with *asCCR* especially affects processes in the cell wall, chromatin, DNA and the chromosome.

The desire to monitor transcript abundance with microarray starting from small amounts of mRNA has led to several protocols for amplification. We compared two amplification methods, PCR and T7-transcription. Amplification by T7-transcription better reflects the variation of the unamplified transcriptome, and gives transcripts with a greater range of lengths, greater estimated mean length, and greater variation of expression levels, but lower average GC content, than those from PCR.

Keywords: acetyl bromide method, Cinnamoyl CoA reductase (CCR), Gene Ontology (GO), lignin, microarray, *Picea abies* [L.] Karst, real-time PCR, thioacidolysis, transgenic, wood

Author's address: Johan Wadenbäck, Department of Plant Biology and Forest Genetics, Swedish University of Agricultural Sciences (SLU), Box 7080, SE-750 07 Uppsala, Sweden. Johan.Wadenback@vbsg.slu.se

Solet quoque post novem annos communis omnium Sueoniae provintiarum sollempnitas in Ubsola celebrari. Ad quam videlicet sollempnitatem nulli praestatur immunitas. Reges et populi, omnes et singuli sua dona transmittunt ad Ubsolam, et quod omni poena crudelius est, illi qui iam induerunt christianitatem, ab illis se redimunt cerimoniis. Sacrificium itaque tale est. Ex omni animante, quod masculinum est, novem capita offeruntur, quorum sanguine deos placari mos est. Corpora autem suspenduntur in lucum, qui proximus est templo. Is enim lucus tam sacer est gentilibus, ut singulae arbores eius ex morte vel tabo immolatorum divinae credantur

(Adam von Bremen, *Gesta Hammaburgensis ecclesiae pontificum*, c. 1070)

Veit eg að eg hékk vindgameiði á nætur allar níu, geiri undaður og gefinn
Óðni, sjálfur sjálfum mér, á þeim meiði er manngi veit hvers af rótum renn.

(*Hávamál*, c. 1270)

Contents

Appendix

The presented thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Wadenbäck, J., Clapham, D., Gellerstedt, G., von Arnold, S. 2004. Variation in content and composition of lignin in young wood of Norway spruce. *Holzforschung*, *58*, 107–115.

II. Wadenbäck, J., von Arnold, S., Egertsdotter, U., Walter, M., Grima-Pettenati, J., Gellerstedt, G., Gullion, T., Clapham, D. Lignin biosynthesis in transgenic Norway spruce harboring antisense constructs of cinnamoyl CoA reductase (CCR). (*Submitted)*.

III. Wadenbäck, U., Egertsdotter, U., von Arnold, S., Göransson, H., Ameur A., Clapham, D. Down-regulation of the gene encoding cinnamoyl CoA reductase affects several genes associated with cell wall function in embryogenic cultures of Norway spruce. (*Manuscript*).

IV. Wadenbäck, J., Clapham, D.H., Craig, D., Sederoff, R., Peter, G.F., von Arnold, S., Egertsdotter, U., 2005. Comparison of standard exponential and linear techniques to amplify small cDNA samples for microarrays. *BMC Genomics*, 6:61 doi:10.1186/1471-2164-6-61.

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Introduction

Norway spruce and its wood have historically played important roles, and persist today even if most people rarely think about it. An example is the Christmas tree. Another is in the production of paper. Indeed, in the beginning of the electronic era, people envisaged a paper-less society, but with the emergence of printers, the development has been quite the opposite. To produce such high-quality paper the wood often comes from Norway spruce. However, in the papermaking process one of the integral components of wood, lignin, needs to be removed. Introducing genes in Norway spruce by genetic engineering offers possibilities to change the content or composition of lignin in wood, thereby reducing the amount of energy and toxic chemicals in pulping.

Conifers and Norway spruce

In mythology and religion

In many religions, the evergreen tree with its cones is the common symbol for eternal life. In ancient Egypt, a pinecone staff was a symbol of the solar god Osiris. The Greeks recognized spruce as being associated with the deity Poseidon, and the god of wine and male fertility; Dionysus and its Roman counterpart; Bacchus are often seen carrying a coniferous tree and a cone staff. However, Greeks such as Theophrastus, whom Carl von Linné called the Father of Botany, did not make the same distinction between conifers as today (Berlin, 1973), making the taxonomic determination difficult. Similarly, there was an unknown evergreen tree next to the temple in Uppsala, used for animal- and human sacrifices (von Bremen, c. 1070). In Rome people associated spruces with grief and put out spruces at the gate when someone in the house was terminally ill. The same practice is found much later in Scandinavia, allegedly to scare off evil spirits. Moreover, a spruce, stripped of bark and branches except for the top, was also placed outside the house hosting a wedding or spruces aligned the road, with their tops broken, pointing towards the cemetery during a funeral.

The Christmas tree is often explained as a Christianization of the ancient pagan idea of the evergreen tree. In addition, the Christmas tree is possibly a modern version of the archetypal Cosmic World Tree found in many Indo-European religions, with a vertical axis reaching from a celestial realm in the far heavens, down through a middle realm and down to an underworld. Indeed, the Roman Catholic Church transferred many of the celebrations of the Roman Saturnalia, such as feasting and exchanging gifts, to Christmas. However, the associations between decorating with evergreen shrubs, a common practice during Saturnalia, and paganism were too evident and therefore forbidden (Tertullian, c. 197). In addition, according to one legend, Saint Boniface attempted to introduce the idea of trinity to the pagan Germanic tribes using the cone-shaped evergreen trees because of their triangular appearance. Even today, Roman Catholicism employs cones in artwork, *e.g.* the Pope carries a pinecone staff and the largest pinecone in the world is found in the Vatican Square. The first Christmas tree in its familiar

form is said to have been erected in Riga in 1510. There are other early references to Christmas trees in the Bremen guild chronicle of 1570 and from Basel, 1597. The Christmas tree remained confined to the upper Rhineland in protestant homes for a relatively long time. It was spread by Prussian officials in the wake of the Congress of Vienna in 1815 among the nobility and to royal courts as far as Russia. In Sweden, the Christmas spruce originally was the same kind of stripped spruce as for weddings and appeared at the beginning of the 19th century, although often as a small one placed on the table. Today 75% of Swedish households have Christmas trees, of which 80% are Norway spruce.

Economic importance

Many coniferous species are of great commercial importance, for timber, pulp, fuel and chemicals, and as ornamentals. Today Norway spruce is used in building and joinery, for pulping where it is particularly favored for the production of high quality paper, for bioenergy, as a source of turpentine, for Christmas trees, and as an ornamental tree in parks and gardens.

Forestry and the forest industry plays an important role in Sweden accounting for almost four percent of Sweden's GDP. The forest industry constitutes 10% and 14% respectively of employment and added value in Swedish industry. In addition, it accounts for more than 12% of the exports (Swedish Forest Industries Foundation, 2004). Sweden ranks fourth among the world's paper and pulp exporters and second in exports of sawn softwood timber. Sweden supplies 13% of the paper demand in Europe and cover about 12% of the total consumption of sawn timber in EU. The Swedish land area is covered to 55% by productive forest (43% pine, 39% spruce, 11% birch) (Swedish Forest Industries Foundation, 2004).

Lignin content largely determines the calorific value of wood as fuel, but has to be removed during pulping for the manufacture of high quality paper. The lignin content and composition is furthermore very important for the quality of wood, which in turn affects *e.g.* the pulp- and papermaking processes. Residual lignin in the wood fibers causes a discoloration and a low brightness level of the pulp. Lignin is therefore removed with more or less damaging procedures to the cellulose component and with the use of environmental unfriendly chemicals and large quantities of energy (Baucher *et al*., 2003; Boudet *et al*., 2003; Chen *et al*., 2001).

Classification

The conifers (*Coniferae*) comprise six to eight families, with a total of 65-70 genera and 600-650 species (depending on taxonomic opinion). They are cone-bearing seed plants with vascular tissue; all conifers are woody plants, the great majority being trees with just a few being shrubs. The pine family (*Pinaceae*) is the largest conifer family in species diversity, with 220-250 species in 11 genera. Typical examples include cedars (*Cedrus*), Douglas-firs (*Pseudotsuga*), firs (*Abies*), larches (*Larix*), pines (*Pinus*) and spruces (*Picea*) (Price, 1995).

The spruce genus (spruce [Polish *z Prus*; 'from Prussia']; *Picea* [Latin *pix*, *picis*; 'pitch, resin']) includes about 35 species, three quarters of which are Eurasian and one quarter North American (Germano *et al*., 2002). One of the most economically important spruce species is Norway spruce (*Picea abies* [Latin *abies*; 'fir, evergreen tree with needles'], less common *Picea excelsa* [Latin *excelsus*; 'tall']).

Distribution

Conifer species can be found growing naturally in almost all parts of the world, and are frequently dominant plants in their habitats. Conifers have their highest species diversity in the mountains of south and west China and Japan (Taylor, Patterson & Harrod, 1994). In the northern hemisphere, they are restricted to subtropical high altitude, temperate, and boreal regions. The principal realm of conifers in the northern hemisphere is the boreal forest known by its Russian word, Taiga, where they are the dominant species across vast tracts of Scandinavia, Russia, Alaska, and Canada. The Taiga is characterized by thousands of square kilometers of relatively few species of plants and animals (Raven *et al*., 1999). The forest is also important for the carbon dioxide balance. Trees absorb vast amounts of carbon dioxide from the atmosphere. In Sweden, the forests are growing faster than they are harvested and therefore the quantity of bound carbon is increasing every year. The two dominating species in Swedish forests are Norway spruce and Scots pine (*Pinus sylvestris*).

Norway spruce is the common spruce of northern Europe; native of Scandinavia, Russia, in the mountains of central Europe, southwest to the western end of the Alps, and southeast in the Carpathians and Balkans to the extreme north of Greece but is not found in the Mediterranean area. Norway spruce is naturally common all over Scandinavia; the northern limit is in the arctic, just north of 70°N in Norway, except in parts of Scania and Denmark. Norway spruce is one of the most widely planted spruces, both within and outside its native range, *e.g.* it is planted in southeastern Canada, northeastern United States, Rocky Mountains, and Pacific Coast region.

Norway spruce is one of the most recent immigrants among tree species in Scandinavia. Fossil records indicate that Norway spruce immigrated from the east and northeast and then spread west- and southwards during the mild climate of the Bronze Age (first millennium B.C.) (Huntley & Birks, 1983). At the beginning of the first millennium, forests of Norway spruce were being established in Sweden, forcing out the previously dominating birch- and pine forests.

The eastern limit of Norway spruce in Russia is hard to define, owing to extensive hybridization with the Siberian spruce (*Picea obovata*), but is usually given as the Ural Mountains. However, trees showing some Siberian spruce characters extend as far west as much of northern Finland, with a few records in northeast Norway. Another spruce occurs rarely in the central Alps in eastern Switzerland. Treated as a distinct species, it takes the name Alpine spruce (*Picea*

alpestris). As with Siberian spruce it hybridizes extensively with Norway spruce; pure specimens are rare.

Norway spruce prefers moist soils in humid, cool, temperate regions and grows best in fertile soil. The roots develop mycorrhizal associations with various fungi. Similar to other spruce trees, Norway spruce avoids arid climates, but it encounters cold stress and some drought stress at alpine timberline sites. It handles shade well and easily out-competes other trees creating a dense forest.

Morphology and life cycle of Norway spruce

Norway spruce is a large, cone-bearing tree with a straight trunk and pyramid-shaped crown of spreading branches. Several deviant forms have been discovered. The tree grows 10 to 55 m tall (average 24 m) with a trunk diameter of up to 1.5 m. The bark of the stem is light brown, smooth on young trees, which is then cracked and scaly turning red-brown in southern Sweden and gray in northern Sweden. The branches are situated in evenly spaced whorls from the stem. The upper branches are directed upwards whereas the lower branches are drooping. Branchlets droop from the branches toward the ground. Twigs on the branchlets are slender, drooping, mostly hairless, and rough with peglike bases. Norway spruce shoots are orange-brown and glabrous (hairless). The needles differ greatly in size, morphology and direction; on a shoot they are 12-30 mm long, stiff, sharp-forward-pointed, quadrangular in cross-section (not flattened), and shiny dark green on all four sides with inconspicuous whitish stomatal lines spreading on all sides of the twig from very short leafstalks. They stay in place for up to 10 years.

All conifers are wind-pollinated. In Norway spruce, the male cones are red or yellow-green, short, small and cylindrical, and bear numerous spirally arranged cone-scales. The male cones have two structures called microsporangia which produce yellowish pollen. To fertilize the ovum, the male cone releases pollen that is carried on the wind in great amounts, in Sweden during May and June to the female cone. The female cones are light red or brown, 9-17 cm long (the longest of any spruce), cylindrical, hanging down when mature and bear numerous thin, triangular-pointed, irregularly toothed cone-scales. When a pollen grain lands near a female gametophyte, it undergoes meiosis and fertilizes the female gametophyte.

In gymnosperms, the overall embryo development can be divided into three stages: (1) Proembryogeny, with a phase of free nuclear division; in *Picea*, there are eight nuclei before cell wall formation. Proembryogeny includes all the stages before the elongation of the suspensor. (2) Early embryogeny, which initiates with the elongation of the suspensor, and includes an interesting phase where a gradient of programmed cell death is established along the cells of the suspensor. It ends with the failure to develop new so-called tube cells within the suspensor. (3) Late embryogeny is a period of active histogenesis including the development of the primary shoot and root meristems and distinct procambium, cortex pith and root cap, and the slow elimination of the suspensor by programmed cell death (Bozhkov *et al.,* 2005). In Norway spruce, the female cones mature 5-7 months after pollination during the autumn. Certain years, cones are particular prolific, so-

called cone-years. During dry weather, the subsequent year after maturing the female cones open their scales and the seeds fall out. The seeds are black, 0.4-0.5 cm, pointed and egg-shaped and are distributed via a small light-brown 15 mm wing, a lamell left from the scale. The seed slowly descends to the ground with the help of the small wing, allowing it to spread over great distances. The seed germinates and grows into a seedling.

The transition from a juvenile vegetative and non-reproductive to an adult reproductive phase is gradual and can take many years, in Norway spruce, typically 20-25 years. Three regulatory genes with potential roles in this juvenile-to-adult transition in Norway spruce seem to be involved: *DAL1*, and two *LEAFY*-related genes *PaLFY* and *PaNLY*. *DAL1* is a MADS-box gene, the activity of which is initiated in the shoots of juvenile trees at an age of three to five years, and then increases with age. In addition, the activity of *DAL1* further shows a spatial pattern along the stem of the tree that parallels a similar gradient in physiological and morphological features associated with maturation to the adult phase. Both *LEAFY*-related genes are expressed throughout the juvenile phase (Carlsbecker *et al*., 2004). Norway spruce trees can be more than 400 years old.

Wood and the cell wall

The cross-section of a tree trunk is composed of four principal tissues: bark, phloem, vascular cambium and xylem. The bark consists of a layer of dead cells that protect the stem from damage and from drying out. The phloem is an inner layer of live bark consisting of cells that transport sugars and other materials made in the foliage to all the other living cells in the tree. The vascular cambium is a 'bifacial' meristem that provides initials (stem cells) from which phloem cells are derived by mainly periclinal divisions on the outer side and xylem cells on the inner side (Schrader *et al.,* 2004). The xylem cells consist of the water-conducting tracheary elements and the ray cells. Owing to the continual division of cells, the cambium layer slowly moves outwards as the tree increases in girth. As the tree expands in girth, the outer bark then splits or is shed and is replaced by the new outer layer. Wood, or essentially secondary xylem, is formed through an ordered developmental process (xylogenesis) involving cell division, cell expansion (elongation and radial enlargement), cell wall thickening (involving cellulose, hemicellulose, cell wall proteins, and lignin biosynthesis and deposition), programmed cell death and heartwood formation (Figure 1). The coordination of secondary cell wall synthesis and programmed cell death begins well in advance of the execution of cell death, with the approximately concurrent commencement of secondary cell wall synthesis and the production of hydrolytic enzymes. Sap water ascends via the xylem. However, our understanding of how wood develops is far from complete.

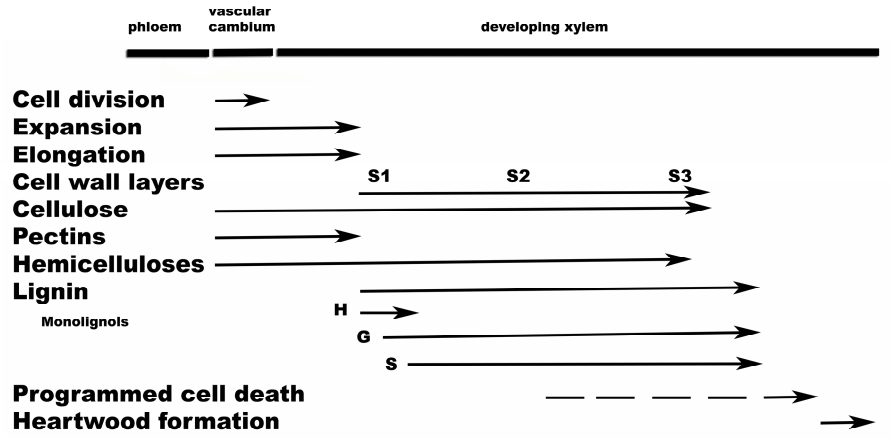

Fig. 1. Wood development in aspen (*Populus tremula*) (adapted after Hertzberg *et al*., 2001a).

Because wood is formed in a variable environment and subject to developmental control, xylem cells are produced that differ in size, shape, cell wall structure, texture and composition (Plomion, Leprovost & Stokes, 2001). Properties of wood include density, hardness, fiber length, flexibility, durability against attack by insects, fungi and other agents and can vary markedly between dried seasoned- and moist freshly-cut wood. The density of wood depends on the thickness of the fiber walls in relation to the size of the cells' hollow centre. Annual growth rings are a feature of trees that grow in climates where growth virtually ceases for part of the year, such as during cold winter months. In spring, when the trees burst into growth, wood is formed relatively rapidly and the earlywood tracheary elements tend to be large and thin-walled, allowing rapid water conduction at a time when the risk of cavitation (breakage of water columns) is minimal. Later in the season, as tree growth slows, the newly formed tracheary elements are of smaller diameter and thicker-walled. The ring width variable in Norway spruce explains the main part of the density variation (Rozenberg, Franc & Cahalan, 2001). There are consequently many different kinds of wood, yet all wood share common features.

Cell walls constitute the main component of wood. Synthesis of cell wall components often represents a great part of the overall cellular carbohydrate metabolism. The three main constituents of the cell wall are cellulose, hemicellulose and lignin, making up the bulk of the lignocellulose matrix. Cell wall metabolisms can be described in terms of two pools of metabolic intermediates linked by reversible reactions. Metabolites can be added to or withdrawn from these pools to serve the needs of various anabolic and catabolic pathways. One of these pools is composed of hexose phosphates, the other of penthose phosphate pathway intermediates, the triose phosphates glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The direction of flow through these pools depends on the requirements of the cell. The biosynthesis of cellulose and hemicellulose is closely linked to the hexose phosphate pool, whereas the lignins derive from erythrose 4-phosphate and phosphoenolpyruvat. In addition, there are

some minor constituents including proteins, lignans, flavonoids, isoflavonoids, tannins etc., which according to their differential deposition contribute to the overall properties of the wood.

Plants, unlike other organisms, have an extra compartment where carbohydrates are metabolized, the plastids. Carbohydrate metabolism in the cytosol is connected with plastid metabolism by a series of carriers in the plastid envelope. Furthermore, plants have inherent flexibility in their metabolism owing to the requirement of the plant to withstand changes in the external environment. Often, several enzymes can bypass an otherwise principal reaction, although the exact control mechanisms often are not completely elucidated. Metabolite pools in the cytosolic and plastid compartments communicate by way of highly specific carrier proteins. The hexose phosphate pool contributes intermediates to glycolysis and many other processes.

As the plant grows, the elongation of the cells depends on the relation between the internal turgor pressure and strength of the cell wall. At the same time, the cell wall must be resilient enough to withstand the pressure it is subjected to transporting water from the roots to the foliage without collapsing. In this, plant cell expansion requires the integration of local wall loosening and the controlled deposition of new wall materials (Shpigel *et al*., 1998) which process is poorly understood.

Different kinds of wood

Sapwood and heartwood

Two main kinds of wood are found in mature trees: sapwood and heartwood. New sapwood is formed by the vascular cambium cells as a tree grows. The sapwood lies in a broad ring around the heartwood and consists of cells that carry water and nutrients upwards from the roots to the needles. The new heartwood cells slowly fill with tannins, resins and other substances making them more resistant to decay and insect attack. Heartwood, is usually the bulk of the cross-section. It helps support the tree and has no role in its growth (Plomion, Leprovost & Stokes, 2001).

The color of wood is determined by various substances, such as polyphenols, which are deposited in the cell walls. In most trees the heartwood is distinctively colored, commonly red, pink, brown or yellow and sometimes even green and purple. However, some woods have pale colored heartwood not easily distinguished from sapwood.

Hardwood and softwood

Angiosperms produce hardwood and gymnosperms produce softwood. A hardwood tree is often, but not necessarily, a harder and denser wood than a softwood tree. The terms come from old logging camp where woods were sometimes named by their resistance to sawing. The hardwood/softwood terminology does make some sense. Gymnosperms do tend to be less dense than

deciduous trees, and therefore easier to cut, while most hardwoods tend to be denser, and therefore sturdier.

Strength in hardwood trees is imparted by fibers, vessels and axial- and ray parenchyma cells. Softwoods are mainly composed of three cell types: tracheids, axial and ray parenchyma cells. Fibers in angiosperms provide mechanical support and make up the bulk of the wood. The bulk (about 90 percent) of softwood is made up of long narrow tracheids, up to four millimeters long that fit closely together. The cells of the vessels in angiosperms are tubular-shaped and conduct water. The conifer tracheids function in both mechanical support and water transport. The tracheids are similar to the fibers in angiosperms but are longer and usually have thinner walls. Pits in the cell walls of the tracheids enable sap to pass from cell to cell as it moves up the stem. The ray cells store nutrients in the stem and are found in all kinds of wood. Unlike the other cells of sapwood which are arranged vertically, ray cells are arranged horizontally, extending radially outwards towards the bark. Often rays are only one cell wide and several cells high whereas in some trees the rays are very large and give the wood characteristic patterns (Sjöström, 1993; Plomion, Leprovost & Stokes, 2001).

The tracheids of both hardwoods and softwoods consist of a primary and a secondary cell wall. The primary cell wall is a thin layer outside the secondary cell wall that consists of three sublayers; S1, S2 and S3 (Timell, 1986) that are created after each other in xylogenesis (Figure 1). Lignin is deposited between the touching tracheid cell walls through a region rich in lignin called the middle lamella which help to hold the tracheids firmly together (Sjöström, 1993). The lignin in the middle lamella also has been observed to have a different chemical structure than the lignin in the secondary cell wall (Meier, 1985).

Reaction wood

When a tree is subjected to an external force such as wind or bending the tree responds by producing reaction wood to restore the displaced stem to its original position. For a hardwood species, this means producing so called tension wood *pulling* on the same side as the external force. Tension wood is rich in cellulose, and has a lower lignin content; the vessels are reduced in number. The tracheids, so-called G-fibers, differ considerably from those of normal wood. In the G-fibers, the normal cell wall layers are partly replaced by the gelatinous G-layer, consisting of highly crystalline cellulose. It is unclear how the structure is related to the pulling force developed (Norberg & Meier, 1966). In softwoods reaction wood is called compression wood and leads to *pushing* on the opposite side compared to tension wood; on the lower side of a branch point or a leaning stem. Compression wood is characterized by a higher lignin- and lower cellulose content making the tissue hard and dense; supporting the tree. The tracheids in compression wood are shorter, thicker and rounder in cross section than normal wood. The S1 layer is thicker than in normal wood and the S3 layer is absent and the S2 layer has helical cavities from the lumen deep into the S2 layer (Fengel & Wegener, 1983; Sjöström, 1993).

Cellulose and hemicellulose

Cellulose is a linear homopolymer built up of β-D-glucopyranose units linked by 1-4 glycosidic bonds in a flattened structure, held together by hydrogen bonds as microfibril bundles present in the cell wall. Cellulose is the dominating component in the cell wall of plants, constituting about 40% of the wood and coupling up to 10,000 monomeric units together, making it Earth's most abundant polymer. The substrate for cellulose synthesis, UDP-glucose, can be formed from hexose phosphatases by UDP-glucose pyrophosphorylase and from sucrose by sucrose synthase. Cellulose is presumably synthesized by an enzyme complex associated with the plasma membrane (Saxena & Brown, 2005).

Cellulose microfibrils confer support especially to young unlignified tissue. The cellulose microfibrils can be aligned by cortical microtubules, but have also been seen to function independently. Microfibrils are arranged locally and nascent microfibrils appear to associate tightly with the plasma membrane. The templated incorporation model postulates that the nascent microfibril is incorporated into the cell wall by binding to a scaffold that is oriented; further, the scaffold is built and oriented around either already incorporated microfibrils or plasma membrane proteins, or both. The role of cortical microtubules is then to bind and orient components of the scaffold at the plasma membrane. In this way, spatial information to align the microfibrils may come from either the cell wall or the cell interior, and microfibril alignment with and without microtubules are subsets of a single mechanism. Taken together, the alignment favors growth in certain directions and constraints in others (Baskin, 2001). Research has been focused on the bacterium *Acetobacter xylinum*, which produces large quantities of cellulose microfibrils without interacting with the cell wall synthesis. These separately produced microfibril ribbons of cellulose are synthesized independently from other polysaccharides, which differ from the mechanism in the plant wall (Saxena & Brown, 2005).

The grouping of polysaccharides into hemicellulose is ambiguous. The name hemicellulose was originally proposed in the late 19th century, for the polysaccharide fraction obtained by extraction with dilute alkaline solution. It was assumed that hemicelluloses were related chemically and structurally to cellulose and possibly intermediates in cellulose biosynthesis. Nowadays, a hemicellulose is defined as any of several heteropolymers, except cellulose and pectin, present in almost all cell walls along with cellulose (Sjöström, 1993). The molecular weight of hemicellulose is usually lower than that of cellulose and hemicellulose have a weak undifferentiated structure compared to that of crystalline cellulose (Shpigel *et al*., 1998). Hemicelluloses are found in the matrix between cellulose fibrils in the cell wall. The components in lignocellulose are tightly associated and in several processes it has been to be difficult to separate hemicellulose from cellulose and lignin without modifying the hemicellulose. The type and amount of hemicellulose varies widely, depending on plant, tissue and developmental stage. In conifers, the main hemicelluloses are glucomannan and arabinoglucuronoxylan (Table 1). In the S2-wall in spruce tracheids the glucomannan is believed to be situated between the cellulose fibrils and the lignin whereas the

arabinoglucuronxylan is found mixed with the lignin (Salmen & Olsson, 1998). Moreover, there seem to be two types of lignin in the cell wall of spruce that are either associated with glucomannan or with arabinoglucuronoxylan (Lawoko, Henriksson & Gellerstedt, 2005). The biological functions of hemicellulose are not fully understood. It contributes to the mechanical properties of the secondary cell wall and interacts with cellulose and lignin, possibly regulating porosity and strength. In the primary cell wall xyloglucan may be crucial in maintaining the three-dimensional conformation via interfibrillar polysaccharide linkages in the cell wall. In addition, being a macromolecule, hemicellulose probably influences the moist content of the living tree.

Table 1. *Carbohydrates in Norway spruce (Picea abies) and birch (Betula verrucosa) wood (adapted from Sjöström, 1993)*

	Weight%	
	Norway spruce	Birch
Cellulose	42	41
Glucomannan	16	2
Arabinoglucuronoxylan	9	28
Other carbohydrates	3	3
Lignin	27	22
Extractives	2	3
Residual components	1	1

Lignin

Lignin (Latin *lignum*; 'wood') is, after cellulose, the most abundant polymer on Earth. The term was coined as early as 1819 by de Candolle. In 1838, Anselme Paye found that wood treated with nitric acid was dissolved and had a higher carbon content than the remaining solid material. This substance was defined as lignin and in 1897, Peter Klason found that lignin was related chemically to coniferyl alcohol. Eventually lignin was recognized as a macromolecular substance of coniferyl alcohols joined by ether linkages (Sjöström, 1993). Owing to its physical properties, lignin has played a decisive role in the adaptation of plants to terrestrial life. It is important in waterproofing the vascular tissue, and is required for the defense against insects and pathogens. Lignin confers rigidity to the vascular tissue and, being hydrophobic, greatly improves water-conducting properties (Rogers & Campbell, 2004). Lignification is one of the plant's defenses to environmental conditions such as wounding and mechanical stresses. The lignification of tissues is also part of the defense arsenal of plants to limit insect and pathogen attack (Lange, Lapierre & Sandemann, 1995).

Lignin is now understood as a complex and variable structure polymerized from phenolic alcohol precursors (monolignols) (Figure 2 and 3). The lignin content and composition vary according to plant group, cell type, cell wall layer and position within the layer (Donaldson, 2001; Peter & Neale, 2004). The synthesis

of lignin is one of the most energy demanding pathways in plants, requiring large quantities of carbon skeletons. The three conventional monomers of lignin, coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, differ by their methylation degree. They give rise to the *p*-hydroxyphenyl- (H), guaiacyl- (G) and syringyl- (S) units of the lignin polymer, respectively (Figure 3). The third monolignol unit is mainly found in angiosperm species and does not occur in coniferous wood. Potentially, lignin rich in H- or G-units contains more carbon-carbon bonds than lignin rich in S units because the aromatic C-3 or C-5 position of the H- or G- unit is free to make linkages (Halpin, 2004).

The lignin of normal conifer xylem consists mainly of G-units, and a much smaller proportion of H-units. The H-units appear to make up only about 1% of the lignin of normal wood, but can contribute 20-70% of the lignin units of the compression wood (Lange, Lapierre & Sandemann, 1995; Gindl, 2002 and *loc. cit.*) that forms on the under side of leaning stems and side branches, or 10-40% of the lignin formed in cell cultures in response to elicitors (Lange, Lapierre & Sandemann, 1995). In angiosperms, the water conductive xylem vessel elements of the secondary xylem and the primary xylem cells are rich in or contain only G lignin, like the tracheids of the gymnosperms, whereas the nonconductive xylem fiber cells are rich in S lignin. The fibers and rays of angiosperm wood, and the middle lamella between fibers, contain G/S-lignin (Anterola & Lewis, 2002). Lignin units are linked by different types of bond within the same lignin macromolecule (Adler, 1977; Hatfield & Vermerris, 2001; Anterola & Lewis, 2002) (Figure 3). The most common linkage is the β-O-4 ether bond, which is degraded in Kraft pulping and bleaching (Chen *et al*., 2001, Anterola *et al*., 2002; Boudet, 2003).

It is thought that H-lignin is initiated at specific sites at the periphery of the S1 sublayer, the first formed layer of the three layers of the secondary wall (Timell, 1986), and progresses into the middle lamella and then is followed by deposition of G- and S-lignin (Anterola & Lewis, 2002 and *loc. cit.*) (Figure 1). The similarity of the lignin in the tracheids of conifers and the vessels of angiosperms implies a strong selective pressure to conserve the pathway of G-lignin biosynthesis in water-conducting cells during land plant evolution (Peter & Neale, 2004).

Biosynthesis and subcellular localization

Despite the effort put into lignin research, the general uncertainty of the biosynthesis has resulted in apparently different models of the lignin pathway in recent years. The formation of lignin is the result of the flux through three biosynthetic pathways (Figure 2). The availability of lignin precursors depends on the presence and activities of the enzymes of (1) shikimate metabolism, (2) the general phenylpropanoid pathway, and (3) the monolignol-specific pathway in the lignifying cell. The monolignols are synthesized beginning from the amino acid phenylalanine through the action of phenylalanine lyase (PAL) (E.C. 4.3.1.5) that catalyses the nonoxidative deamination of phenylalanine to cinnamic acid and NH_3. The second enzyme is cinnamate 4-hydroxylase (C4H) (E.C. 1.14.13.11)

which controls the conversion of cinnamic acid into *p*-coumaric acid. The third enzyme is 4-coumerate CoA ligase (4CL) which catalyses the formation of coumaroyl CoA from *p*-coumaric acid. The fourth step involves the enzymes *p*-coumarate 3-hydroxylase (C3H) and the novel *p*-hydroxycinnamoyl CoA shikimate/quinate *p*-hydroxycinnamoyltransferase (HCT) (Hoffman *et al*., 2003), a member of a large family of acyltransferases which is involved in the convertion of *p*-coumaroyl shikimate and *p*-coumaroyl quinate into caffeoyl CoA. The next enzyme is caffeoyl CoA 3-*O*-methyltransferase (CCoAOMT) (E.C. 2.1.1.104) which catalyses the methylation of caffeoyl CoA to ferulolyl CoA. The last two enzymes are cinnamoyl CoA reductase (CCR) (E.C. 1.2.1.44) which catalyzes the conversion of cinnamoyl CoAs (feruloyl CoA and *p*-coumaroyl CoA) to their respective cinnamaldehydes (coniferaldehyde and *p*-coumaraldehyde) and the first enzyme of the monolignol specific part of the lignin biosynthetic pathway, and cinnamyl alcohol dehydrogenase (CAD) (E.C. 1.1.1.195) which reduces the cinnamyl aldehydes into their corresponding alcohols (coniferyl alcohol or *p*-coumaryl alcohol). The caffeic acid *O*-methyltransferase (COMT) (E.C. 2.1.1.68) and ferulate 5-hydroxylate (F5H) have the predominant role of producing S-lignin, and therefore seem not to be involved in lignin biosynthesis in conifers (Anterola *et al*., 2002).

There is still a great deal of uncertainty as to the subcellular localization of the lignin biosynthetic enzymes. The sequences of many of the lignin biosynthetic genes do not contain any signal sequences or other targeting domains which indicates that they are cytosolic (Halpin, 2004 and *loc*. *cit*.). Three of the lignin biosynthetic enzymes, C4H, C3H and F5H (all P450 enzymes) are however clearly localized on membranes, but their exact location within the cell has not been determined. On the other hand there have been reports of possible enzyme:enzyme complexes that might be membrane bound, channeling phenylpropanoid intermediates (Dixon *et al*., 2001; Gou, Chen & Dixon, 2002). The transport of monolignols to the cell wall has been proposed to be in the form of glucosides (Dharmawardhana *et al*., 1999; Lim *et al*., 2001), in vesicles associated with highly developed trans-Golgi network (Samuels *et al*., 2002) or across the plasma membrane directly with or without a specific transporter (Halpin, 2004). Two plausible models can therefore be outlined for conifers. The simpler model is based on free diffusion of substrates between enzymes in the cytoplasm, the membrane bound enzymes C4H and C3H, and monolignol products diffusing through the plasma membrane to the cell wall. The more advanced model utilizes the possibility of metabolic channeling with membrane bound enzyme:enzyme complexes. The monolignols are subsequently glucosylated and transported in vesicles to the cell wall.

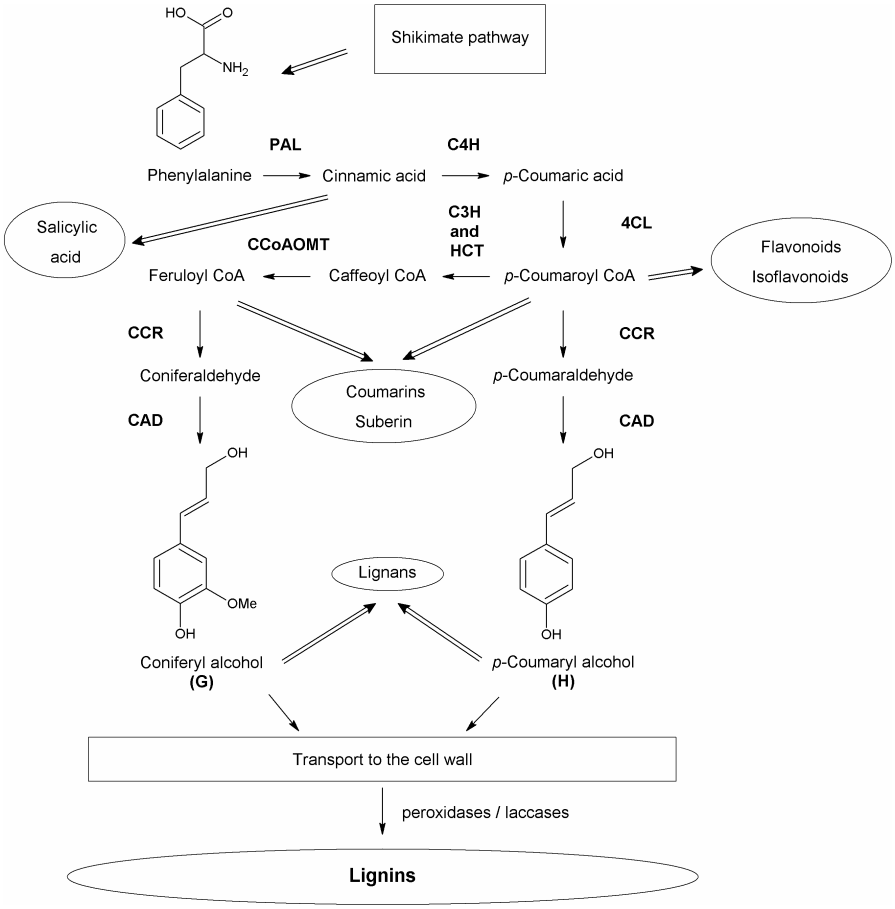

Fig. 2. Lignin biosynthesis in conifers (adapted after Humphreys & Chapple, 2002; Peter & Neale, 2004; Rogers & Campbell, 2004).

Finally monolignols are thought to be polymerized to lignin in a peroxidase- (E.C. 1.11.1.7) and/or laccase- (E.C. 1.10.3.2) catalyzed radical reaction, although the multitude of these enzymes has made it difficult to investigate properly. Concerning the design of macromolecular lignin configurations *in vivo* there are two opposing views. The first, classical, view is that lignin is randomly assembled and from monomeric precursors (Freudenberg & Neish, 1968), a truly random spontaneous phenomenon (Hatfield & Vermerris, 2001). The lignin composition is therefore not strictly controlled but depends on the precursors delivered to the lignifying zone. However the dehydrogative polymer obtained synthetically does not have the same ratios of covalent linkages as the native lignin (Terashima *et al*., 1996), which has meant that this model has been questioned. The other view suggests that there is full biochemical control of the metabolic pathways (Gang *et al*., 1999). This is supported by the findings of 'dirigent' proteins in the cell wall and possible lignin initiation sites (Lewis & Davin, 2000). The mechanism for dirigent proteins is known for the synthesis of lignans that are formed from monolignols (Davin *et al*., 1997) and a homolog for the dirigent proteins has also

been found in poplar coinciding with lignification (Hertzberg *et al*., 2001a). This view has been heavily contested by the fact that lignin is racemic whereas lignin formed by dirigent proteins is expected to be optically active (Ralph *et al*., 1999). Furthermore, proteins may be sterically blocked from diffusing into the lignified cells. In addition, new proposals involving redox shuttle-mediated oxidation has been presented that need further scrutiny (Önnerud *et al*., 2002).

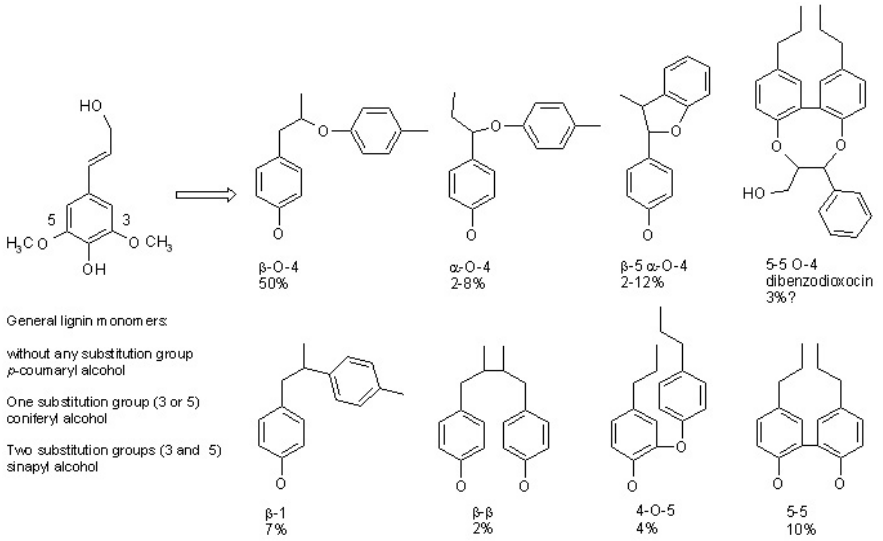

Fig. 3. Proportion of different types of linkages connecting the monolignol units in lignin (adapted after Sjöström, 1993).

Cellular regulation

Many of the enzymes involved in lignification are regulated at the transcriptional level. Furthermore, there is growing evidence for the coordination of lignin biosynthetic gene transcription (Raes *et al*., 2003; Rogers & Campbell, 2004). Possible regulatory mechanisms include transcription factors, regulatory elements, regulation via the circadian clock and coexpression of genes. The activity of transcription factors such as those of the MYB family (Hertzberg *et al*., 2001a; Patzlaff *et al*., 2003) that can activate transcription targeting AC elements residing in promotor regions essential for expression in vascular tissues (Lacombe *et al*., 2000; Raes *et al*., 2003; Rogers & Campbell, 2004). In loblolly pine (*Pinus taeda*) the mechanism regulating this includes PtMYB4, which binds to the AC cis-elements in promoters of genes encoding enzymes of lignin synthesis (Patzlaff *et al*., 2003) and in white spruce (*Picea glauca*) several additional MYBs have been found and are currently under investigation (John MacKay's lab, personal communication). In addition, other elements such as H- and G-boxes and *cis*-elements, are located in lignin biosynthesis genes, possibly associated with stress and wounding responses (Lauvergeat *et al*., 2002). Moreover, in Arabidopsis (*Arabidopsis thaliana*) transcript abundance of monolignol synthesis genes displays diurnal rhythm in relation to photoperiod that persists as a circadian rhythm under continuous light (Harmer *et al*., 2000). Finally, it has been shown in

Arabidopsis and rice (*Oryza sativa*) that there are chromosomal regions about 100 kb long, which display coexpression of lignification genes (Ma & Tian, 2005).

Breeding of Norway spruce

Conventional approaches

The first societies for forest tree breeding in Sweden founded in 1936 (for South Sweden) and 1941 (for North Sweden), merging in 1959 and later evolving into the present tree-breeding branch of Skogforsk. Much of the initial efforts were spent on hardwood and ideas picked up from agricultural experiences. The reorganization into a national forest tree-breeding institute in 1967 was more directed towards conifer breeding. The first basic plus-tree selections were carried out in the period 1940-1960 in mature wild forests.

Tree breeding and improvement programs have indeed made great gains in productivity and quality over the last 40 years, despite that traditional breeding of Norway spruce is a very slow process. Typically, one breeding cycle takes 20 to 25 years. The improvement is obtained through well-known quantitative genetic techniques involving recurrent testing, selection and crossing. Testing and selection is based on specific traits identified in the breeding program. Selected plants which best fulfill the requirements are then used for further breeding and for mass-propagation. In conventional breeding programs, by the time the superior genotypes have been identified in field trials, they are too old to be propagated vegetatively. Consequently, the identified genotypes are lost and can only be used as grafts for establishing seed orchards. The possibility to propagate spruce vegetatively creates significant advantages both for deployment of selected genotypes through mass-propagation and for capturing and enhancing the genetic gain in the breeding program.

Somatic embryogenesis and genetic engineering

A plant reproduces naturally through the development of zygotic embryos. Formation of the embryo begins with the division of the fertilized eggs or zygote within the embryo sac of the ovule. Through an orderly progression of divisions, the embryo eventually differentiates, matures, and develops into a new plantlet. Alternatively, the plant can be derived from a single somatic cell or a group of somatic cells. This regeneration process, called somatic embryogenesis is a method for vegetative propagation of trees (Figure 4) with many advantages over propagation through rooted cuttings. Somatic embryogenesis initiated from immature seed embryos has the potential to produce infinite numbers of somatic plants via *in vitro* tissue culture where all the plantlets produced have the same genetic makeup. It has been shown that somatic embryogenesis combined with cryopreservation is an attractive method to propagate Norway spruce vegetatively (Högberg *et al*., 2001). If genotypes that go to field tests in the breeding program are cryopreserved, the elite genotypes identified during the field trials can be thawed and mass-propagated for use in reforestation programs. Forestry based on somatic embryogenesis has indeed a high potential to become a valuable tool for intensive wood production. Consequently, more efficient land use practices might

be adopted where high production in certain areas can be combined with conservation efforts in other areas.

Genetic engineering enables new genes to be added into selected elite genotypes without changing other properties. This gives the breeders the opportunity to improve economically important traits, which cannot be modified by conventional methods within a reasonable period. The full potential of genetic engineering will only be achieved with the integration into conventional breeding programs. The multitude of traits to be improved using genetic engineering includes altered lignin properties for reducing down-stream processing costs. However, more information about the genetics and the biochemical and physiological pathways involved in specific traits is required. Transgenic trees are very valuable for studying the regulation of different traits. Furthermore, transgenic trees can be used for identification of candidate genes for use in molecular breeding.

Transgenic plants

Three types of genetic transformation systems are used with higher plants. Two systems include vectors based on naturally occurring plasmids of *Agrobacterium tumefaciens* or on plant viruses. The third are physical methods for introducing DNA fragments into the target cells. *A. tumefaciens* infect plants with a specialized plasmid that harbors a segment, T-DNA, with tumor–inducing genes, which can be replaced by any other gene of interest, resulting in stable integration of the gene. Another commonly used method involves bombardment of cells with high-velocity microprojectiles (gold or tungsten), that have been coated with the gene of interest (Klein *et al*., 1987; Christou *et al*., 1990; Ahuja, 2000).

The first transformed conifer with stable expression of a transgene was reported by Ellis *et al*. 1993. Since then the number of genetically engineered conifer species has gradually increased. However, the development of techniques for conifers has been slow due to, in part, difficulties in genetic transformation such as to maintain the regeneration-capacity of the transformed plant material (Robertson *et al*., 1992). Transgenic Norway spruce plants have been produced both via *A. tumefaciens* (Wenck *et al.,* 1999; Klimaszewska *et al.,* 2001) and by microprojectile bombardment (Walter *et al*., 1999; Clapham *et al*., 2000; Clapham *et al*., 2003). Each method has advantages and disadvantages. Stable agrobacterial transformation is more efficient, at least with selected genotypes, and transgene copy numbers are marginally lower and the integration pattern simpler, at least in comparison with plants produced by bombardment with whole plasmids. Unwanted DNA is however, often incorporated from the vector backbone outside the T-DNA region, and at frequencies of 30-60% reported for tobacco and Arabidopsis (Wenck *et a*l., 1997). Particle bombardment is less dependent on the genotype of the recipient cells; and after bombardment with isolated gene cassettes rather than entire plasmids, only the genes of interest are incorporated into the transgenic plants, with predominantly simple integration patterns (Fu *et al*., 2000, Altpeter *et el*., 2005). Particle bombardment, unlike agrobacterial methods, can be used to transform plastids and mitochondria (Altpeter *et al*., 2005 and *loc. cit.*).

Lignin engineering

An attractive objective in tree breeding is to reduce the content of lignin, or alter its composition, to favor delignification in Kraft pulping. Of particular interest are cases in which lignin content is reduced and cellulose is increased. To be realistic the changes must occur without seriously impairing essential functions in the tree. A renewed interest in the lignification process emerged about 15 years ago in molecular biology, genetic engineering and with improved analytical techniques. The genes that have up to date been targeted in different plant species include PAL, 4CL, HCT, COMT, CCoAOMT, CCR, CAD, F5H and MYB factor genes. Both lignin content and composition have been altered in this way (for reviews see *e.g.* Baucher *et al*., 2003; Boerjan, Ralph & Baucher, 2003; Halpin, 2004; Rogers & Campbell, 2004). Which enzymatic steps are suitable for down-regulation, if the intention is a reduction in lignin content, and/or increase the extractability of lignin without seriously impairing plant growth and vitality? It is primarily dependent on the desired reduction in lignin content and secondly, in the composition of lignin. However, suppression of CAD, CCR or 4CL and overexpression of F5H have so far shown the most promising results (Halpin, 2004 and *loc*. *cit*.).

In angiosperms there are numerous studies of both natural mutants and transgenics that underexpress genes of monolignol biosynthesis. The first report of a genetically modified conifer was that of a naturally occurring loblolly pine tree mutant. This tree had a mutation of the gene encoding CAD reducing its activity to 50% of the homozygous wild-type in heterozygotes and to 1% in mutant homozygotes (Sederoff *et al*., 1999). In the heterozygotes, the content of lignin is reduced by only about 1%, but the composition is altered in several respects and in 4-6-year-old plants, the delignification efficiency is increased up to 20% compared to wild-type without serious reduction in pulp yield (Dimmel *et al*., 2002; Gill, Brown & Neale, 2003). In the mutant homozygotes, dihydroconiferyl alcohol, a minor component of most types of lignin, is incorporated at 10-fold normal levels, together with other normally minor components such as coniferaldehyde, and vanilin. The wood is brown colored. Although there is an increase in the proportion of condensed bonds, the molecular size of the lignin molecules appears to be 35% reduced, which could explain the relative ease of delignification (Dimmel *et al*., 2001). The volume growth of the 4-6-year-old trees heterozygous for *cad-n1* was enhanced 14%, whereas growth of trees homozygous for the mutation was impaired.

The reaction catalyzed by CCR is considered the first step committed to monolignol synthesis (Lacombe *et al*., 1997; Peter & Neale 2004; Li *et al*., 2005). In tobacco (Piquemal *et al*., 1998; Ralph *et al*., 1998; Lauvergeat *et al*., 2001; O'Connell *et al*., 2002), poplar (Baucher *et al*., 2003) and Arabidopsis, plants with down-regulated CCR activity have been produced, with various degrees of reduction in lignin content and effects on growth. There have been reports of tobacco (Piquemal *et al*., 1998; Ralph *et al*., 1998; O´Connell *et al*., 2002) and Arabidopsis (Jones, Ennos & Turner, 2001; Goujon *et al*. 2003a) plants often with stunted growth and collapsed vessels with mutated or suppressed CCR and a 50%

reduction in lignin content. The collapsed vessels walls are explained to be due to loosening of the secondary cell walls and an increase in condensed lignin units (Chabannes *et al*., 2001a, b; Pinçon *et al*., 2001; Goujon *et al*., 2003a). Both tobacco and Arabidopsis plants with residual CCR activity have fewer β-O-4 bonds (O´Connell *et al*., 2002; Goujon *et al*., 2003a), a decrease in the non-condensed fraction of G-lignin compared to S-lignin (Piquemal *et al*., 1998; O´Connell *et al*., 2002; Goujon *et al*., 2003a) and contains sinapic and ferulic acids (Goujon *et al*., 2003a, b). In addition, inhibited CCR activity in tobacco can result in the incorporation of unusual constituents such as tyramine ferulate (feruloyl-tyramine) (Ralph *et al*., 1998). The occurrence of these unusual constituents may account for the brown coloration found in xylem cells of CCR-dowregulated tobacco (Piquemal *et al*., 1998). Tobacco plants with suppressed CCR also had a larger proportion of free phenolic groups in the non-condensed lignin and improved paper pulp characteristics (O'Connell *et al*., 2002). There are also different expressions of some CCR-genes reported in Arabidopsis, which would imply that different CCR-genes are responsible for constitutive lignification, whereas other are enhanced due to infection (Lauvergeat *et al*., 2001).

Homozygous tobacco plants, down-regulated in either CAD or CCR, have been crossed (Chabannes *et al*., 2001a). In the double heterozygous offspring, it was shown that suppression of both genes had a synergistic effect in lowering lignin content. The lignin structure in the offspring was however more similar to the wild-type than any of their CAD or CCR deficient parents without apparent adverse effect on phenotype. Stacking of transgenes has otherwise been performed in tobacco down-regulated for COMT 1 and/or CCR, which showed an enhancement in expression of CCR in COMT 1 antisense plants, but not *vice versa* (Pinçon *et al*., 2001). The effects of CCR suppression appeared to dominate with lower lignin content and increased S/G ratio. For the double transformants, the expression of CCR or COMT 1 depended on the level of down-regulation of each enzyme, suggesting a certain degree of crosstalk between some genes in the lignin biosynthetic pathway. Furthermore, epitope distribution showed an altered pattern, especially in vessels, after immunolabeling of non-condensed G or S units. Some cell wall loosening was also reported in the antisense *CCR* plants but not in the antisense *COMT 1* plants.

Aims of the present study

The overall aim of this study has been to investigate the potential for changing the amount and/or composition of lignin in Norway spruce by using a transgenic approach. Lignin content and composition of plants transformed with an antisense construct of a gene encoding CCR, an important enzyme of lignin biosynthesis, have been compared with that of transformed and untransformed controls. In addition, since it is important for practical forestry that other characters such as phenology are not altered unfavorably, attention was given to this.

To estimate how meaningful it is to study lignin characters in juvenile plants, lignin content and composition were compared in one-year-old phytotron-grown plants and nine-year-old trees grown in a plantation. Also related to 'early selection', possible changes in gene expression detectable even at the embryogenic callus stage were studied by microarray techniques. Since the quantities of RNA required for creating target for hybridization to microarrays are often limiting, methods for amplifying target were compared.

Results and discussion

Variation in lignin in young Norway spruce (Paper I)

Variation in wood properties has long been a concern of the forest industry and factors that reduce variation are regarded as highly desirable because of the gains involved in optimization (Zobel & van Buijtenen, 1989). Up to now, mainly variation in wood density and tracheid length have been studied, but very little is known about the natural variation in content and composition of lignin. In recent years much research has been concerned with the modification of lignin in plants by genetic transformation or by selection of genetic variants. However, before applying genetic engineering, it is crucial to have information about the natural variation within and among populations and how it changes with age. In this study, we compared the lignin content and some aspects of lignin composition in 1-year-old plants and young 9-year-old trees of Norway spruce belonging to the same full-sib families. Lignin content was analyzed in 71 plants and 40 trees derived from 10 families, and lignin composition in 40 plants and 40 trees derived from four families.

The lignin content was assessed according to the modified acetyl bromide method (Dence, 1992; Fukushima & Dehority, 2000; Hatfield & Fukushima 2005) which works by the formation of acetyl derivatives of unsubstituted OH groups within a lignin polymer and bromine replacement of α-carbon OH groups rendering the lignin molecule soluble in acetic acid. The analysis is essentially done spectrophotometrically from the absorbance at 280 nm, contributed by the aromatic rings, of the resulting solution. The information about the lignin composition was obtained by using thioacidolysis, a well-established degradation

analysis method for the semi-quantitative estimation of arylglycerol β-aryl ether structures (often called β-O-4 linkages) in lignin (Lapierre & Monties 1986; Lapierre & Rolando, 1988; Rolando, Monties & Lapierre, 1992; Bardet *et al*., 1998; Önnerud, 2002). The reaction consists of treating lignin with boron trifluoride and ethanethiol using dioxane as the solvent. The phenylpropane units (H, G or S) are then substituted with three thioethyl groups in the aliphatic side-chains at position α, β and γ. These monomers correspond to non-condensed β-O-4 structures in the lignin and are quantified using gas chromatography (GC-FID and GC-MS) on their silylated derivatives. The content of monolignols (C9-units) was assessed by comparing the thioacidolysis results with the total amount of lignin. Traditionally, in measuring C9-units in Norway spruce by thioacidolysis, the only component measured is G lignin, owing to the overwhelming dominance of G-lignin in the lignin matrix. The G-lignin fragmentation pattern produces a main fragment of 269 m/z, corresponding to 63% of its prominent fragments, whereas an H-unit produces a main fragment of 239 m/z, corresponding to 48% of its prominent fragments (Rolando *et al*., 1992). We found that a small proportion, 1.5%, of the G-lignin consistently disintegrates into smaller fragments with a size of 239 m/z which happens to be the size of the main fragment of H-lignin. By comparing the total ionic chromatogram (TIC), the chromatogram at 239 m/z and the fragmentation ratios of G- and H-units we could therefore calculate the H-lignin content indirectly despite the low concentration of H-lignin in normal Norway spruce wood.

The lignin content was lower for the plants, 27.4%, and higher for the trees, 31.4%, compared to the mature reference material, 28.1%, and reported values of Norway spruce (Timell, 1986 and *loc*. *cit*.). The coefficient of variance was higher for the plants than for the trees. However, the lignin content did not vary significantly within or among different families as 1-year-old plants or as 9-year-old trees and the standard error within families decreased with age. The number of C9-units g/l lignin, ranges from 516 to 1186 μmol C9-units g/l lignin in plants and from 716 to 953 μmol C9-units g/l lignin in trees, with no significant differences among the families. However, the interaction between family and age was significant. The H-lignin was on average 0.6 percentage units lower in the plants than in the trees (0.8% versus 1.4%).

There are few studies of genetic variation in lignin content among families or provenances of Norway spruce. The phenotypic variation found has been small (Hannrup *et al.*, 2004). Lignin content in Norway spruce grown in Sweden varies very little with temperature and altitude, from 27% to 29% of wood dry weight (Nylinder & Hägglund, 1954). As regards genetic variation, for a random sample of 9-year-old clones of Sitka spruce, the clonal mean for lignin content was between 24.4% and 28.2% (Silva, Wellendorf & Pereira, 1998). Our results generally show a higher lignin content for the young trees and lower for the plants but we could not detect a significant difference among individuals in the full-sib families. We assume that the amount of compression wood and thus H-lignin is usually higher in trees growing in the field than in plants growing under controlled conditions.

After thioacidolysis, the silylated derivatives of the H-, G- or S-units are present in two forms, the *erythro*- and *threo* stereoisomers of the β-O-4 linkage (Brunow *et al*., 1993; Helm & Li, 1995; Bardet *et al*., 1998) visible in the chromatogram as a double peak. The *threo* contribution to the G-lignin double peak was considerably lower in both the plants and the trees than for the reference material. *Threo* content in lignin did not vary among families or between plants and trees. However, the interaction between family and age varied significantly in a similar manner as for the C9-units. This trend could also be observed in the double peak of the H-lignin. The ratio between the *erythro* and *threo* stereoisomers depends mostly on the steric configuration of the reactants and pH (Brunow *et al*., 1993). The *erythro* and *threo* stereoisomers have slightly different chemical properties, and thus pulping capabilities, where the *erythro* forms cleave faster than their *threo* counterparts (Obst, 1983; Jiang & Argyropoulus, 1994). In most previous thioacidolysis studies, the focus has been on mature material of Norway spruce where the contribution of *erythro* and *threo* stereoisomers is very similar, but in this study, the *erythro* and *threo* stereoisomeric ratios are strikingly different. The lower *erythro* contribution in our material might indicate that at least in young material, the *erythro* stereoisomer is incorporated preferentially in the lignin structure.

In conclusion, the amount and composition of lignin does not vary within or among the families at the same age. However, the amount of lignin increases with age while the change in lignin composition interacts with family and age.

Evaluating transgenic *asCCR* plants (Paper II)

An attractive objective in tree breeding is to reduce the content of lignin, or alter its composition, to facilitate delignification in pulping. The changes must however occur without seriously impairing essential functions in the tree such as growth and development. In general, the lignin content of spruce shows low phenotypic and genetic variation, of the order of 3-4% (Silva, Wellendorf & Pereira, 1998; Hannrup *et al*., 2004). The aim of this study was to elucidate the possibilities to change the lignin in Norway spruce by taking a transgenic approach.

One isoform of *CCR* was isolated from the cambial sap of a mature Norway spruce tree. Embryogenic cultures of Norway spruce were transformed by a particle bombardment method essentially as described in Clapham *et al*. (2000) with the Norway spruce *CCR* gene fused in antisense orientation to the maize ubiquitin promoter *UBI1*. The transformed control carried the *GUSA* gene driven by a tomato *LHC* promoter. The plasmids in both cases contained a *BAR* gene cassette for selection on glufosinate, driven by a separate *UBI1* promoter. In addition, both gene constructs had a *NOS* terminator. Plants were regenerated (Clapham *et al*., 2000) and grown in a greenhouse placed in three randomized blocks together with untransformed control plants. Shoots were collected from five-year-old plants during the maximum elongation period for screening of transcript abundance. Plants from eight transgenic *asCCR* lines together with transformed- and untransformed controls were selected for lignin analysis. Measurements of lignin content and composition were assessed by the acetyl bromide method and thioacidolysis essentially as described in Paper I.

Fig. 4. Transgenic Norway spruce from embryogenic cultures to plants. A) Somatic embryogenic colonies. B) Regenerated somatic plantlets. C) Three-year-old *asCCR*-plants in the greenhouse.

To study the expression of specific genes quantitative real-time PCR is a powerful methodology. Primer pairs for quantitative real-time PCR for *PAL*, *C4H*, *4CL*, *C3H*, *COMT*, *CCoAOMT*, *CCR* and *CAD* were selected; all with efficiency values above 1.9. In addition, several potential reference genes of Norway spruce were evaluated for stability (Vandesompele *et al*., 2002), chosen from the most diverse biosynthetic pathways available excluding genes in the shikimate metabolism, general phenylpropanoid-, and monolignol specific pathways.

Generally, there are two strategies of quantification: an absolute and a relative quantification. In absolute quantification, the RNA or DNA copy-number is determined by the comparison with a calibration curve. Having some advantages, this approach requires a lot of space on a typical real-time plate, therefore compromising the amount of wells available for samples. Furthermore, there is always a risk of over- or underestimating gene expression due to differences both between the samples as well as between the samples and the calibration curve. The relative quantification method, often referred to as the $2^{-\Delta\Delta CT}$ method, is based on the expression ratio of the target gene versus a reference gene to investigate the changes in gene expression levels (Gentle, Anastasopoulos & McBrien, 2001; Livak & Schmittgen, 2001; Pfaffl, 2001). Absolute quantification was used in this work to estimate the copy-number of transgenes in the genome of the each transgenic line. In short, it is performed by a dilution series of the same plasmid used for the transformation (linearized with *Sac*I) with or without a background of genomic DNA from the untransformed control. All comparisons of the copy-number of *asCCR* and *BAR* were normalized within each 96-well plate to the copy-numbers of the reference genes *phosphoenolpyruvate carboxylase* (*PEPC-1*) and *α-tubulin* (*Tubulin*), together with one transgenic line used as a calibrator between plates. All the comparisons for transcript abundance were made using the relative quantification approach with *PEPC-1*, *Tubulin* and *histone H2A* (*Histone*). For *BAR* and *NOS,* the copy-number assessments were 30% lower in the presence than in the absence of background DNA. For *asCCR*, the background DNA disturbed the linear relationship between plasmid concentration and amplification by PCR, presumably because *asCCR* primers bound to the background DNA; the relationship was linear in the absence of background DNA. Therefore, for accurately determining transgenic copy-numbers, it is important to use background

untransformed genomic DNA in PCR, and adjust for possible interactions with endogenes that might appear.

Transgene copy-number was in general low, 1 to 8 copies except for one line which contained 30-40 copies of the transgenes. There is good agreement between the number of *asCCR*- and *BAR* copies for each line both within and between embryogenic cultures and plant shoots. Since a low transgene copy-number is usually considered desirable, the relationship between copy-number and transcript abundance of *BAR*, *asCCR* and the endogenous *CCR* gene was studied. Transcript abundance of *BAR* and *asCCR* increased up to about 3 to 4 copies and then declined. In addition, the bioassay with glufosinate showed that needles from all the transgenic lines except one were tolerant.

In general the transcript abundances of *asCCR* in the different lines were similar to the transcript abundances of *BAR*. There was a strong positive correlation ($r \geq 0.81$) between the transcript abundance of *BAR* and *asCCR*. The transcript abundance of *asCCR* was up to 11% of the transcript abundance of sense *CCR* in the transgenic lines. In plants from the two strongly down-regulated lines, A78:2 and A78:21, the *CCR* transcript abundance was up to 50% lower than in the control plants. Pairwise correlations of transcript abundance for the genes available of lignin biosynthesis were calculated for the transgenic *asCCR* lines as well as the control. Using quantitative real-time RT-PCR, transcript abundance was determined for *CCR* and seven other genes of monolignol biosynthesis. The transcript abundance of the monolignol synthesis genes, measured over the various lines, was in many cases significantly correlated with the transcript abundance of *CCR*, with the genes encoding enzymes acting at the end of the reaction pathway *CCoAOMT, CCR* and *CAD* showing the strongest correlation ($r \geq 0.89$). The transcript abundance of *COMT* was however not significantly correlated with that of any of the other genes except *C4H*.

The total lignin content in plants of the transgenic lines was 3.6% lower than plants of the control group. No general correlation between transcript abundance of *CCR* and the total lignin content was observed. The H-lignin content in the transgenic lines was 14.8% lower than the control. The H-lignin proportion of total lignin was strongly correlated with the total lignin content ($r=0.83$). The total lignin and the amount of non-condensed H-lignin in plants from the lines with significantly down-regulated *CCR*, A78:2 and A78:21, were 5.3% and 23% lower, respectively, than in the control plants. Samples from the two most down-regulated lines and the controls were subjected to small-scale Kraft pulping. The pulp material was air-dried for kappa number (SCAN-C 1:00) and viscosity measurements (SCAN-C 15:88). The kappa number (a measurement of deligninfication) was not significantly reduced in the down-regulated lines, despite the lower lignin content. If the kappa number was related to the lignin content rather than the dry weight of pulp, then the value increased 5% in the group of A78:2 and A78:21 relative to the control group. This is consistent with more condensed lignin, and/or lignin of higher molecular weight, in the plants with down-regulated *CCR*. Measurements of viscosity to assess the degree of cellulose degradation by pulping did not display any significant differences. ^{1}H-^{13}C cross-

polarization, magic-angle spinning (CPMAS) experiments were obtained with 3 sec recycle delays and 1 ms CP contact times. After normalizing the NMR-spectra the most striking feature was the variation of the double peak at 30 ppm. It showed a weak negative correlation with the total lignin content (r=-0.60) and the amount of H-lignin (r=-0.51). Verification of the double peak being associated with fatty acids, such as possibly the aliphatic part of suberin, was made using the desuberization procedure described in Lopes *et al*. (2000).

Antisense *CCR* lines of Arabidopsis contained up to 50% less lignin, accompanied by an apparent reduction in content of H-units, loosening of the secondary cell wall structure and incorporation of ferulic acid in the cell wall (Goujon *et al*., 2003a, b). There have been reports of dwarf plants associated with down-regulation of *CCR* in tobacco (O'Connell *et al*., 2002) and in Arabidopsis with mutated *CCR*; with collapsed vessels, reduction in lignin content and stunted growth, reduced apical dominance, abnormal leaf shape, and chlorosis (Piquemal *et al*., 1998; Goujon *et al*., 2003a). Furthermore, a relative increase in condensed lignin has been shown in both tobacco (Chabannes *et al*., 2001a, b; Pinçon *et al*., 2001) and Arabidopsis (Goujon *et al*., 2003a) down-regulated in *CCR*. If relatively fewer H-units are synthesized in Norway spruce, we should expect to observe an increase in the transcript abundance of *C3H* and *CCoAOMT* relative to both *C4H* and *4CL*. However, there is a tendency of a decrease. This is consistent with the view that H-units are increasingly incorporated into condensed lignin, rather than synthesized in reduced amounts. Lüderitz & Grisebach (1981) found that spruce CCR is active on both substrates, *p*-coumaroyl CoA and feruloyl CoA, but the former is a poor substrate for the enzyme, which partly explains the normally low content of H-units in spruce lignin.

In tobacco plants strongly down-regulated in CCR activity, unusual amounts of cell wall bound phenolics have been found together with a tendency to an orange-brown coloration in the xylem probably due to these unusual components (Piquemal *et al*., 1998; Ralph *et al*., 1998). Similar results have been noted for Arabidopsis (Goujon *et al*., 2003a). A few of our acetyl bromide preparations displayed red coloration but we did not observe unusual color in the xylem, preparations, or notably adverse effects on growth and wood anatomy, in the more moderately (50%) down-regulated *CCR* plants of Norway spruce. Furthermore, in NMR studies, we observed an enhanced peak at around 30 ppm. The fact that the 30 ppm significantly decreased after the methoxide/methanol treatment is consistent with the presence of fatty acids. The peak may correspond to what has been identified as the aliphatic part of suberin (Lopes *et al*., 2000; Preston & Forrester, 2004). Interestingly, the aromatic part of suberin is thought to be feruloyl tyramine, as reported in transgenic tobacco, and apparently many of the enzymes used in its biosynthesis are the same as for lignin biosynthesis, with some of the intermediates, feruloyl CoA and *p*-coumaroyl CoA, acting as common substrates in the diverging monolignol- and suberin specific pathways. Our data showed a weak negative correlation between the amount of H-lignin and the enhanced peak. This indicates, together with the highly significant correlation of total lignin and apparent H-lignin, that suberin production might be increased if lignin synthesis is reduced.

Transgene copy-number, preferred to be low, was in accord with our earlier results (Clapham *et al*., 2000; Elfstand *et al*., 2001) and not appreciably higher than reported for *Agrobacterium*-mediated transformation of spruce species (Wenck *et al*., 1999; Klimaszewska *et al*., 2001). Transgene expression seems to be stable over at least five years in Norway spruce, extending earlier observations (Brukhin *et al*., 2000; Clapham *et al*., 2003).

Stem width was measured as an indication of wood volume, and days to budburst in the greenhouse were taken as an aspect of phenology of significance for frost tolerance. In the two lines of Norway spruce with about 50% reduced *CCR* activity and reduced lignin content, A78:2 and A78:21, the stem widths were not significantly different from those of the control group of plants. No significant differences for stem increase among the individual lines were observed. These non-significant effects on growth between our Norway spruce lines are in contrast with the study in the loblolly pine *CAD* mutant, where the gene was associated with increased growth; especially stem radial growth (Wu *et al*., 1999). Budburst was somewhat early in A78:2 but not significantly earlier in A78:21. This may affect decisions as to where the tree of a particular line should be planted in the field to avoid frost. Interestingly there was a strong positive correlation of number of days to budburst with the transcript abundance of *CCR* ($r=0.82$) among the lines.

In conclusion, the antisense method was successful in down-regulating *CCR* in the present study with Norway spruce. Transgene copy-number was in general low and the transcript abundance of *CCR* in the group of seven experimental lines was significantly reduced relative to that of the group of controls. In two lines the transcript abundance of *CCR* was up to 50% reduced, and the corresponding reductions in lignin content were up to 5.3%. We have shown that down-regulation of *CCR* decreases not only the total lignin content but also the apparent H-lignin fraction of the total lignin in Norway spruce. The ratio of H-lignin to G-lignin, as inferred after thioacidolysis, was reduced by 23% in both lines. Since H-units have been proposed to mainly be involved in carbon-carbon linkages (Anterola & Lewis, 2002), it could be hypothesized that a higher proportion of H-units are incorporated in the condensed lignin fraction, thus explaining the reduced recovery of H-units after thioacidolysis from the transformants. The transcript abundances of the various lignin biosynthetic genes were positively correlated, with the transcripts of enzymes at the end of the biosynthetic pathway showing the highest degree of correlation.

Global profiling of *asCCR* embryogenic cultures (Paper III)

We have shown in the previous paper (Paper II) that Norway spruce plants expressing a native gene encoding CCR in antisense orientation displayed decreased *CCR* transcript abundance, lowered lignin content, altered lignin composition and down-regulation of several genes of lignin biosynthesis. From a practical point of view it would be of great advantage to be able to identify important changes in lignin biosynthesis already in somatic embryos of Norway

spruce before plants are regenerated. The aim of this work was to examine the possibility of detecting altered transcript abundance of possible marker genes; in particular those of lignin biosynthesis, already in embryogenic cultures. For this purpose we used transgenic lines that were shown to have lowered *CCR* transcript abundance at an embryogenic stage.

The *asCCR* lines were some of the embryogenic cultures from which the corresponding plant lines of Paper II were derived. The embryogenic lines were transformed with a plasmid construct containing an *asCCR* gene driven by an ubiquitin promoter and a *BAR* gene, for selection on glufosinate, driven by a separate ubiquitin promoter. Both cassettes have a *NOS* terminator. RT-PCR analysis showed that there was detectable transcript abundance of *asCCR* but no detectable transcript abundance of sense *CCR* in three *asCCR*-lines. The embryogenic lines acting as controls were transformed by particle bombardment with a construct containing a *BAR* gene driven by a ubiquitin promoter and a *GUSA* gene driven by chitinase promotor that did not display any expression for GUS during proliferation (Wiweger, 2003; Wiweger *et al*., 2003). In addition, the untransformed control line was included in the analysis. All lines, including control lines, proliferated fast, showed a similar morphology during proliferation and produced high yields of mature somatic embryos after maturation treatment.

The microarray intensity data were normalized using a mixed model system (Brazma & Vilo, 2000; Jin *et al*., 2001; Wolfinger *et al*., 2001; Stasolla *et al*., 2004a, b). The loblolly pine ESTs present on the slide were compared to protein databases (Altschul *et al*., 1997) and coupled to an Arabidopsis identification number. The ESTs were furthermore coupled to Gene Ontology (GO) (Ashburner *et al*., 2000) to describe gene products in terms of their associated biological processes, cellular components and molecular functions and allowed us to extract intrinsic functional information from hundreds of significantly differentially expressed genes.

In order to elucidate if the effect of down-regulation of *CCR* on lignin biosynthesis can be studied in embryogenic cultures, we compared transcript abundances of genes tentatively involved in lignin biosynthesis among the three *asCCR*-lines and the transformed- and untransformed control lines. In contrast to our earlier findings in Norway spruce plants, we could not see any general change of the transcript abundance of the lignin biosynthetic genes besides *CCR* in the *asCCR* embryogenic cultures. The only significantly differentially expressed genes in the *asCCR*-lines putatively associated with lignin biosynthesis were the up-regulation of a PAL and the down-regulation of two peroxidases. A COMT gene was also up-regulated even though it is doubtful if COMT is involved in lignin biosynthesis in conifers (Peter & Neale, 2004). Based on theses results, we do not recommend screening for changes in the expression of lignin biosynthetic genes in embryogenic cultures. An important point to bear in mind is that a small difference between two lines in the transcript abundance of a particular gene does not necessarily imply small biological significance, since the gene product may be produced in limiting quantities that regulate a biological process. Similarly, a large difference in transcript abundance may have little biological effect, if the gene

product is normally produced in excess and does not regulate a biological process (although it may be essential for the process). This is getting more evident when using several *asCCR*-lines from separate transformation events with multiple biological replicates of each line. By using multiple biological replicates and *asCCR*-lines transformed the same way you gain more general or true effects, coupled to the *asCCR*-transgene itself.

The ESTs, that displayed significantly differential transcript abundance when *asCCR*-lines were compared with transformed- and untransformed control lines, were organized into subcategories. According to the GO categorization, main increased subcategories were biological processes such as response to abiotic or biotic stimulus, response to (oxidative) stress, signal transduction, developmental processes, protein complex assembly and nucleosome assembly. The cellular components involved were cell wall, Golgi apparatus, plastid, nucleus and nucleosome. The molecular functions were transferase activity, transcription factor activity, structural molecule activity, DNA or RNA binding, aspartate transaminase activity, hydrolase activity acting on ether bonds and antioxidant activity. In addition, subcategories increased in other biological processes, cellular component unknown and other molecular functions. When comparing both the *asCCR*- and the transformed control lines with the untransformed control line the main increased subcategories were biological processes such as transcription, growth and secretion. The cellular components involved were cell wall, mitochondria and cytosol. The molecular functions were hydrolase activity, transferase activity transferring one-carbon groups, peroxidase activity and (guanyl) nucleotide binding. In addition, subcategories increased in other biological processes, other cytoplasmic components, other cellular components, other molecular functions and other binding.

Interestingly, changes in cellular components related to the cell wall were found both when the three *asCCR*-lines were compared to all the control lines, as well as when transformed lines were compared with the untransformed line. However, since different genes are found in the two comparisons, we assume that the *asCCR* effect on the cell wall is different from the general transformation effect. There were, however, indications of up-regulation in the shikimate pathway when the three *asCCR*-lines were compared to all the control lines. Two 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases, the first enzyme in the shikimate pathway (Entus, Poling & Herrmann, 2002) were affected in an antagonistic manner to each other and two chorismate synthases (Macheroux *et al*., 1999) were up-regulated. Two out of three benzylic ether reductases were also up-regulated, indicating changes in the reductive processes in 8–5-linked lignans, *e.g.* with dehydrodiconiferyl alcohol (Min *et al*., 2003). Peroxidases are involved in several processes (Passardi *et al*., 2005) including lignin biosynthesis. Two peroxidases were significantly down-regulated in the *asCCR*-lines. There seems to be a reduction in cellulose production as cellulose synthase was down-regulated. Furthermore the down-regulation of two UDP-glucose glucosyltransferases (Kleczkowski *et al*., 2004; Lim & Bowles, 2004) and β-tubulin (Lloyd & Chan, 2002) is consistent with a decrease in cellulose production and deposition. Unlike cellulose, other polysaccharides are formed in the Golgi apparatus and are

exported to the external surface of the plasma membrane in Golgi vesicles. Interestingly there was an overrepresentation of Golgi-apparatus-associated GO categories in the *asCCR*-lines. Furthermore, responses to abiotic and biotic stimuli as well as stress were over-represented in the *asCCR*- lines. The heteropolysaccharide, xyloglucan, is a major hemicellulose in primary cell walls of higher plants. The enzyme, xyloglucan endotransferase (XET) can cleave and rejoin xyloglucan chains (Barrachina & Lorences, 1998). Two XETs were found to be differentially displayed in the *asCCR*-lines.

In conclusion we have shown that, down-regulation of CCR does not significantly affect the transcript abundance of genes regulating lignin biosynthesis in embryogenic cultures. However, several genes regulating different pathways associated with lignin biosynthesis and cell wall were affected. Functional assignment defined by GO terms, showed that transformation with *asCCR* affects a broad spectrum of functional categories, especially processes taking place in the cell wall. Furthermore, many changes in genes related to chromatin, DNA and chromosome take place.

Amplification of targets for microarray analysis (Paper IV)

The analysis of transcript abundance in samples of total RNA using microarrays requires microgram quantities of total RNA. However, it is often inconvenient or impossible to obtain sufficient quantities. Therefore, the need to perform microarray experiments with small amounts of tissue has led to the development of several protocols for amplifying the target transcripts. The use of different amplification protocols, however, could affect the comparability of microarray experiments. Amplification of cDNA by a standard PCR procedure (Saiki *et al*., 1988) may result in the differential amplification of particular transcripts, since sequences differ in the rate with which they can be amplified by PCR (Lockhart & Winzeler, 2000). To minimize this problem, the sequences to be amplified can be limited to about 300 nucleotides (Hertzberg *et al*., 2001b) or the concentration of deoxynucleotides in the PCR reaction mixture can be limited (Iscove *et al*., 2002). An alternative approach is linear amplification by *in vitro* transcription from a T7 phage promoter (van Gelder *et al*., 1990). Linear amplification has been shown to retain the relative frequencies of transcripts with reasonable fidelity over a wide amplification range (Wang *et al*., 2000a; Baugh *et al*., 2001; Gomes *et al*., 2003; Schneider *et al*., 2004). However it has been reported that PCR amplification requires less RNA, is more reproducible and generates better target transcripts than linear amplification (Iscove *et al*., 2002; Klur *et al*., 2004). In this study, we mainly compared expression data from loblolly pine cDNA microarrays using transcripts amplified either exponentially by PCR or linearly by T7 transcription. Starting with small amounts of secondary xylem tissue, we compared PCR and T7 RNA polymerase amplification methods directly to investigate if, and how, the biases differ from each other. In addition, we also compared unamplified target versus amplified target for both techniques.

Polyadenylated RNA (mRNA) was extracted from cryotome sections through the cambial region of loblolly pine. The mRNA samples were reverse-transcribed and

the resulting cDNA were amplified by a) exponential PCR amplification or b) linear T7 amplification. The PCR products were directly labeled by Klenow with Cy™ dUTPs. The T7 amplification aRNA products were reverse-transcribed with aminoallyl-modified dUTPs (Sigma, St. Louis, MO, USA) and labeled by coupling to free Cy™ dyes. The consistency of each method was assessed by dividing the samples into two technical repeats. The microarray intensity data was normalized using a mixed model system (Brazma & Vilo, 2000; Jin *et al*., 2001; Wolfinger *et al*., 2001; Stasolla *et al*., 2004a, b). The fold change values were used to divide the genes with significant differential transcript abundance in two groups depending on sign: the PCR and the T7 group. The absolute values (*i.e.* a rescaling of the data disregarding the sign) were then used in the subsequent statistical analysis. Transcripts possible to detect on the microarray were analyzed for sequence length, estimated based on the full length Arabidopsis (www.arabidopsis.org) homolog sequences; GC content as well as abundance.

The correlation of expression intensities between technical repeats was high for both PCR- and T7 amplification ($R^2 = 0.98$) whereas the correlation of expression intensities using the different methods was considerably lower ($R^2 = 0.52$). Correlations of expression intensities between amplified- and unamplified transcripts were intermediate ($R^2 = 0.68–0.77$). For unamplified- versus PCR amplified target lower abundance transcripts were under-represented and highly expressed transcripts were amplified better than average. The differences between unamplified and T7 amplified targets indicates that bias exists when using T7 amplified relative to unamplified targets, especially for highly expressed transcripts.

About 14% of the ESTs on the arrays showed differentially displayed abundance between the two amplification methods with slightly fewer ESTs in the PCR group than in the T7 group. The arithmetic mean of abundance in the PCR group was higher than the T7 group and the PCR group had higher variance. There was a 60% greater variance in length with almost 3 times the maximum length of genes in the T7 group compared to that of the PCR group. The estimated mean length of transcripts present was 27% greater in the T7 group than the PCR group and had about 2 percentage units lower GC content.

It appears that transcripts with a high GC content are amplified faster by PCR than by T7, often overriding the effect of length. If the GC content is nearer the average, long transcripts are favored by T7 amplification. The GC effect is presumably explained by the temperature of extension, which is 68–72°C for Taq polymerase and 37°C for T7 polymerase; high temperature favors polymerization through GC-rich areas. Evolution has in general tuned the cellular machinery, including polymerases, to fit the temperature environment of an organism. This might be reflected in the GC content and the temperature environment of the original organism for each polymerase.

Taken together we have shown that the two main approaches to amplification of small amounts of RNA for microarray studies, PCR and T7 transcription, both introduce bias compared to the unamplified target, and that the nature of the bias is

different for each method. Amplification by the T7 amplification method gives transcripts with a greater range of lengths, greater estimated mean length, and greater variation of abundance levels, but lower average GC content, than those from the PCR amplification method. Therefore, when working with loblolly pine transcriptome, or consequently other transcriptomes with similar GC content, T7 amplification is a better choice than PCR. Amplification with T7 transcription will better reflect the variation of the unamplified transcriptomes than PCR based methods owing especially to the better representation of long transcripts. If transcripts of particular interest are known to have high GC content and are of limited size, however, PCR based methods may be preferable.

Future perspectives

By reducing lignin content and/or increasing the non-condensed fraction of lignin in wood, the amount of energy and environmentally harmful chemicals can be reduced during pulping for the manufacture of paper. In addition, growing Norway spruce as a raw material for bio-fuels is a conceivable future goal, coinciding with the inevitable shortage of fossil fuels. It is, however, unclear how a change of lignin would influence such a production. If the objective is to produce ethanol or methanol, the lignin would probably be a problem in the process whereby a lower, more extractable lignin would be beneficial. On the other hand, because the lignin represents a large part of the stored energy in wood, you would like to leave these components in the fuel, whereby a more- and/or energy-rich lignin in the wood would be preferable. Taking environmental considerations into account the exhaust would probably be cleaner using the first approach producing only water and carbon dioxide. However, everything depends on future biotechnological and chemical advances.

It is clear that more research is needed to relate properties of different kinds lignin-associated genes and gene variants and of lignin itself in various plants, cell types and their function in the plant to effectively be able to develop more precise lignin-engineering strategies. As an alternative approach, conifer research could learn from *e.g.* plant species such as the gnetophytes, especially the tree species found in the gnetales (*Gnetum*). Recent phylogenetic developments of gnetophytes, show a closer relatedness to conifers than previously thought; and in contrast to conifers, they have vessels and produce the more readily extractable S-lignin found in angiosperms. Furthermore, it might be possible to produce Norway spruce plants with more reduced CCR activity and much less lignin than the plants presented in this study. However, it is unclear how a more extreme reduction in lignin content would influence the growth of Norway spruce. As of now, modest lignin reduction may be as interesting as more extreme lignin reduction for practical forestry.

Future projects to change lignin and/or the non-condensed fraction of lignin in Norway spruce might be to modify the expression of several biosynthetic genes at once. This could perhaps be achieved by manipulating the right kind of transcription factor(s) (such as MYB-factors) and/or by stacking several lignin transgenes (perhaps siRNA-*CAD* and/or siRNA-*CCR*; preferable driven by a wood-specific promotor) at once. Considering the recent phylogenetic developments of gnetophytes, with their closer relatedness to conifers, it may also be possible to introduce genes (perhaps lignin specific *COMT* and *F5H*) that could induce S-lignin formation in Norway spruce.

Early prediction of wood and lignin properties is one of the keys for optimizing breeding. Another key is the elucidation of the ‘flowering’ system in Norway spruce, to be able to incorporate the improved material early into the conventional breeding programs. By defined genetic backgrounds-, growth conditions-, emergence of QTL mappings- and, transcriptomics there are great possibilities of

finding marker genes for marker-aided selection of wood and lignin traits in Norway spruce. However, this could only be achieved with improved analytical techniques for small samples of wood. Having this there might indeed be a possibility of detecting wood and lignin changes, at least in seedlings. The problem is to decipher how representative the produced lignin is to that produced in normal mature wood.

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Acknowledgements

This adventure started slightly more than five years ago. A quite handsome Scanian boy applied for position at Ultuna, Uppsala, in the centre of Sweden; the land of the historical archenemy. The boy was not a biologist or similar, like the majority of the other employees, and found the prospect of working with trees slightly daunting, but interesting. In time, he would even have the possibility to learn some Linnaean terminology he thought. Despite the endless ocean of possibilities, certain individuals might have argued that he did not know the consequences of his action. Other individuals might have shrugged their shoulders in utter despair. Even others might even have cried: "Hunta di mi di pågablära! När ska du hitta itt riktet jubb?"

Without further a due, there is, indeed, a multitude of people to thank for making this thesis possible, and I will regrettably probably forget someone. I especially want to extend my heartfelt regards to my excellent main supervisor, David, who always had time for my sometimes strange and sometimes even stranger ideas. Your wonderful creative expressions in Swedish *e.g.* "det är rakt fram" (it's straightforward); I have really taken to heart. Our driving experiences in Raleigh and Atlanta are something else, which I probably never will forget. Other great regards go to the professor of all professors, Sara, for all her great helpfulness and positive energy, although she did not officially have a supervisor responsibility. Regardless of poor or unexpected results, she always manages to turn it around and make you feel better, even though she at times is slightly overly optimistic to achieve results. I regret not having said this earlier: both of you are the greatest I cannot thank you enough. My other regards are to my other assistant supervisors, Björn (a fellow Scanian in exile) and Göran (the wood man). Moreover, I surely want to thank all my wonderful roommates that had to put up with me during these years. Andreas (Hipp! hipp!, respect!), you are a trooper, let us hope the stock market goes up again. Emma (halva garderoben på sig på vintern), you came as a fresh breeze from the west. As a fellow engineer, you always have good arguments. Lyuba and Greg, I wish you all the best and thank you especially for our political discussions.

Secondly, I would like to thank people associated with the Genetic Centre. Thank you all in old Forest Genetics ('the other side' or 'the dark side') like Sam, Rachel, Peter, Kjell, Lada, Hartmut, Nandy, Inger, Malin, Mrudul, Sanna etc., we did a great deal of keeping the name in spite of Plant Biology being so much larger. More thanks go to the rest of the members in our big happy family at Plant Biology and Forest Genetics: Abdul, Andrea, Anna, Anna, Anders, Birgitta, Björn, Carina, Christer, Derek, Dixan, Elke, Eva, Folke, Gun, Guillermo, Gunilla, Harald, Helena, Helena, Helfried, Henrik, Inez, Janne, Jenny, Jennie, Jens, Jens, Jesper, Joel, Johan, Johan, Jon, Kjelle, Lisa, Lotta, Luyba, Magnus, Maria, Marie, Mattias, Mattias, Mats, Mona, Monica, Nadiva, Per, Sara, Sara, Susanna, Tina, Ulrike and Yvonne. Undoubtedly, the social activities at GC have been very important; the Tuesday-innebandy, with and without the magnificent BMC-contribution and WESE with the hard-core team, you know who you are, what

should I have done without that? Thank you all a million for making my stay at the Genetic Centre so enjoyable!

For the help with lignin- and microarray analysis I want to thank Hans and Andrea, and Hanna and Adam who have been crucial. Thank you all the guys currently- or formerly working in Raleigh, Atlanta and Blacksburg: Ron, Len, Debbie, Mattias and Jonas. I especially want to thank Ulrika. We had some really nice time driving around; I hope we can do that again soon. Recently, here in Québec I want to thank Sebastien, John, Hélène, Andreas, Anka, Dragos etc; and especially Carmel who so painstakingly and patiently has been correcting my French.

There is no way that I could ever forget the ULS Postgraduate Council. Harald, Jennifer, Daniel, Johanna we sure had a lot of fun! The meetings were never too long were they? The meetings in Docentnämnden might not have been the most fun in the world though.

On a personal level, I want to thank my mother and father, my sisters and their families respectively. Annika, Melwin and soon alien number two, have really put this work in perspective and at the same time much easier to bear. Thank you also Henrik for our long political discussions. Thank you also Dowile.

In conclusion, a recipe for a successful completion of a doctoral thesis could be something like the following. First of all, manage to collect a good set of supervisors and preferably a good subject. See to that you collect large quantities of confidence in whatever you do, you will need it sooner and more often than you think. In your quest it is important that you have as much backing as you can from your surrounding. Working hours and free time it is all a blur, so get use to it. It is good to try and separate the two if possible but difficult. In addition, make up routines that you can stick to, it really helps you cope with both the hardships and successes. In the final stages of your work, let several people read your thesis drafts for constructive criticism (thank you Elke, Emma, Mats, David, Sara, Andreas and Piddor). Finally, I do not know what I would had done during these years, especially at the finalizing stage of the thesis without the Thai-place, Syltan, Gallan, Findus- and Dafgårds lunches, Sushi Fly in Place Laurier, Subway, Sushi Shop, Star Café, Pepsi Max, French discussion classes, internet, e-mail, Photoshop, Avanza, Sydsvenskan, Radio Français International, Messenger, barbecue on the patio, flæske-svær, freshly-squeezed orange juice, Brita-filter and Dennis benmärgskorvar.

Star Café, la Ville de Québec, Canada, August 7, 2006

 (Codex runicus, c. 1300)