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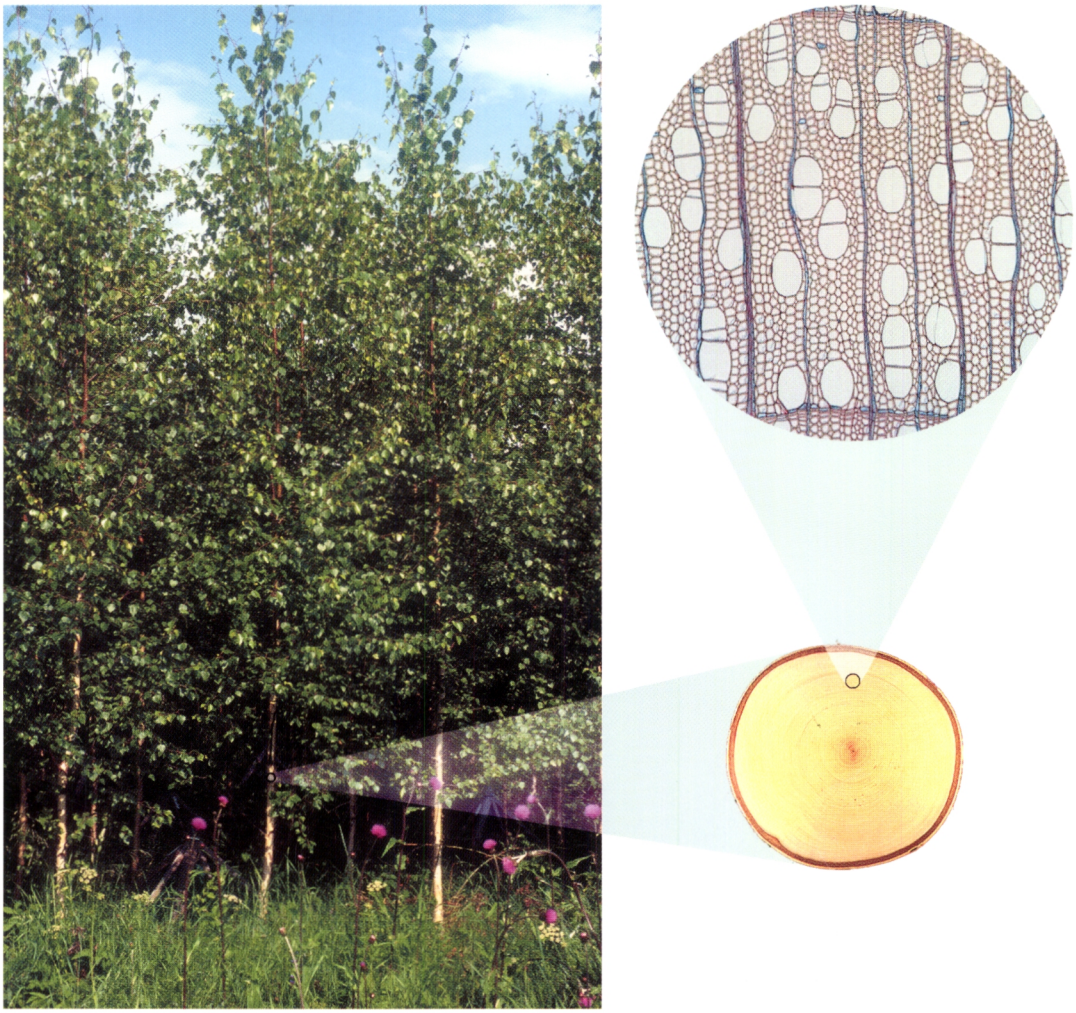

Factors affecting silver birch (*Betula pendula* Roth) wood structure and chemistry - growth hormone genes, defoliation, within-stem and seasonal variation

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Front cover: Seven-year-old silver birch (*Betula pendula* Roth) clone (papers III and IV) and a transverse section of silver birch. Photos: Pekka Saranpää /METLA

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Contents

List of abbreviations

ANOVA	analysis of variance
CaMV	cauliflower mosaic virus
CWI	cell wall index
FA	free fatty acids
FAME	fatty acid methyl esters
GAs	gibberellins
GC	gas chromatography
IAA	indole-3-acetic acid, auxin
IBA	indole-3-butyric acid
LPE	lysophosphatidylethanolamine
MS	mass spectrometry
NPA	N-1-naphtylphtalamic acid
PA	phosphatidic acid
PC	phosphatidylcholine, lecithin
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
pI	isoelectric point
PI	phosphatidylinositol
SPS	sucrosephosphate synthase
SuSy	sucrose synthase
TG	triacylglycerol
TLC	thin-layer chromatography
TSS	total soluble sugars

Summary

Wood structure and chemistry of young and mature Silver birch (*Betula pendula* Roth) trees were investigated in three different studies. The effects of *aux* and *rol* gene transformation on wood structure were studied in 5-year-old greenhouse-grown silver birches (*Betula pendula* Roth) and the effects of defoliation and fertilisation on wood structure in field-grown silver birch clones. The purpose of the transformation and defoliation studies was to find out, how the changes in growth rate affect wood structure and chemistry. Growth of the silver birches was affected by growth hormone genes, defoliation and fertilisation. The *aux* and *rol* genes are known to influence the hormone biosynthesis of plant cells. These *Agrobacterium* pRiA4 plasmid genes can be expressed in plant cells due to their eucaryotic type of regulation. Silver birches with *rol* genes had a typical bushy phenotype, produced less xylem, broke bud later, had shorter vessels and a slightly higher lignin concentration than the control birches. Silver birches with both *aux* and *rol* genes resembled the control birches. In a field experiment, complete defoliation decreased the annual ring width of the birches for at least two years after the treatment. Continuous fertilisation increased the annual increment at all defoliation levels. Gene transformation and defoliation lead to decreased silver birch vessel lumen diameter. However, vessel diameter was shown to increase from the pith towards the cambium. In the defoliation experiment, the vessel proportion was largest in samples taken close to the pith and followed the general pattern presented for silver birch in several earlier works. In contrast, the transformed birches had the smallest vessel proportion and this was shown to be a primary effect of the transformation and not a secondary effect caused by growth reduction. This work shows that the maturation process and growth rate should be taken into account, when the effects of treatments, like transformation, defoliation and fertilisation, on wood structure are studied.

Wood extractives, which comprise a wide range of secondary compounds, are responsible for carbon bound energy storage, act as defence compounds and accumulate in high concentrations during heartwood formation. In this thesis the seasonal turnover and within-stem variation of extractives in young and mature wood of silver birch were studied. At the beginning of new growth, wood extractives are important storage compounds for deciduous trees. The lowest total soluble sugar concentrations occurred in mid-summer and the highest during dormancy. The triacylglycerol (TG) concentration remained at the same level throughout the year and was relatively low, compared to that in other fat-storing trees. However, the neutral lipid fraction contained high concentrations of a number of isoprenoid compounds and, unlike the storage fat fraction, the sterol/isopronoid fraction proved to be biochemically mobile. In mature birches, the between-tree variation of non-structural carbohydrates and lipids was relatively large, especially in wood located above crown height, where the metabolic activity is high due to the proximity of photosynthesising leaves. However, the TG concentration was highest close to the pith, and soluble sugars showed an opposite pattern in that they increased towards the cambium. This work shows that heartwood is not formed in intact mature silver birch stems.

List of original publications

This thesis consists of an introductory review followed by four research articles. In the review the papers are referred to using Roman numbers (I-IV). The papers are reproduced with the permission of the Publishers concerned.

Paper I Piispanen, R., T. Aronen, X. Chen, P. Saranpää and H. Häggman. 2003. Silver birch (*Betula pendula*) plants with *aux* and *rol* genes show consistent changes in morphology, xylem structure and chemistry. Tree Physiology 23:721-733. Heron Publishing - Victoria, Canada.

Paper II Anttonen, S., R. Piispanen, J. Ovaska, P. Mutikainen, P. Saranpää and E. Vapaavuori. 2002. Effects of defoliation on growth, biomass allocation, and wood properties of *Betula pendula* clones grown at different nutrient levels. Canadian Journal of Forest Research 32:498-508. NRC Research Press – Ottawa, Canada.

Paper III Piispanen, R. and P. Saranpää. 2001. Variation of non-structural carbohydrates in silver birch (*Betula pendula* Roth) wood. Trees – Structure and function 15:444-451. Springer-Verlag – Heidelberg, Germany.

Paper IV Piispanen, R. and P. Saranpää. 2004. Seasonal and within-stem variation of neutral lipids in silver birch (*Betula pendula* Roth) wood. Tree Physiology (in press). Heron Publishing - Victoria, Canada.

The doctoral candidate is responsible for writing the results and discussion parts concerning wood properties in papers I and II. The doctoral candidate is responsible for method development and data processing concerning wood structure and chemistry in papers I and II. The doctoral candidate is responsible for writing, data handling and method development in papers III and IV.

1. Introduction

Secondary xylem i.e. wood is a product of the vascular cambium (Larson 1994). The term *cambium* is applied to two lateral secondary meristems. These are the *vascular cambium*, which produces secondary vascular tissues; and the *cork cambium* or *phellogen*, which produces phellem and phelloderm (Romberger et al. 1993). In this thesis the term cambium is used for vascular cambium, a tissue comprising meristematic cells organised in radial files that differentiate centripetally into secondary xylem cells and centrifugally into secondary phloem cells (Larson 1994, Mellerowicz et al. 2001). Wood is a vascular tissue and a secondary tissue, which can be produced in stems, branches and roots, and thus defined as *stem wood*, *branch wood* and *root wood*, respectively.

1.1. The overall anatomical structure and general basic properties of silver birch wood

Silver birch (*Betula pendula* Roth) is a deciduous hardwood tree species, native to temperate and boreal regions of the Northern Hemisphere (Kubitzki et al. 1993). In Europe silver birch has a native distribution from Pyrenees, British Isles and Dolomites to northeastern parts of Europe (Jalas and Suominen 1976). The secondary xylem of silver birch consists of earlywood and latewood vessel members, fibres, and radial and axial parenchyma cells formed from xylem fusiform and isodiametric initials produced by the cambium. Like a distinct diffuse-porous angiosperm, no difference in silver birch wood structure in transverse sections can be detected under the microscope by eye between earlywood and latewood within one growth ring (Figure 1). In addition, idioblasts that contain crystals can sometimes be found in the secondary xylem of silver birch. Each cell type has their determined physiological and structural function in the living tree. The silver birch vessels have the basic function as water transporting elements, while the fibres are more important for support as in other diffuse-porous angiosperm tree species (Tyree and Zimmermann 2002). The secondary xylem of silver birch is formed of typical diffuse-porous angiosperm wood showing no difference in the diameter in transverse direction between early and late wood vessels within one growth ring (Fabisiak and Helińska-Raczkowska 1997). However, in angiosperms with distinct growth rings such as in silver birch, a definite increase in fibre length has been found from the first formed earlywood to the last formed latewood within one growth ring (Bisset and Dadswell 1950). In silver birch fibre length ranges from 0.6 mm to 1.4 mm (Bonham and Barnett 2001). The length of vessel members in silver birch ranges from 0.6 to 0.73 mm (Bruun and Slungaard 1959, Bhat and Kärkkäinen 1981b) and vessel occurrence from 9.4 to 21.5 percent of total volume (Kujala 1946, Ollinmaa 1955, Bhat and Kärkkäinen 1981a).

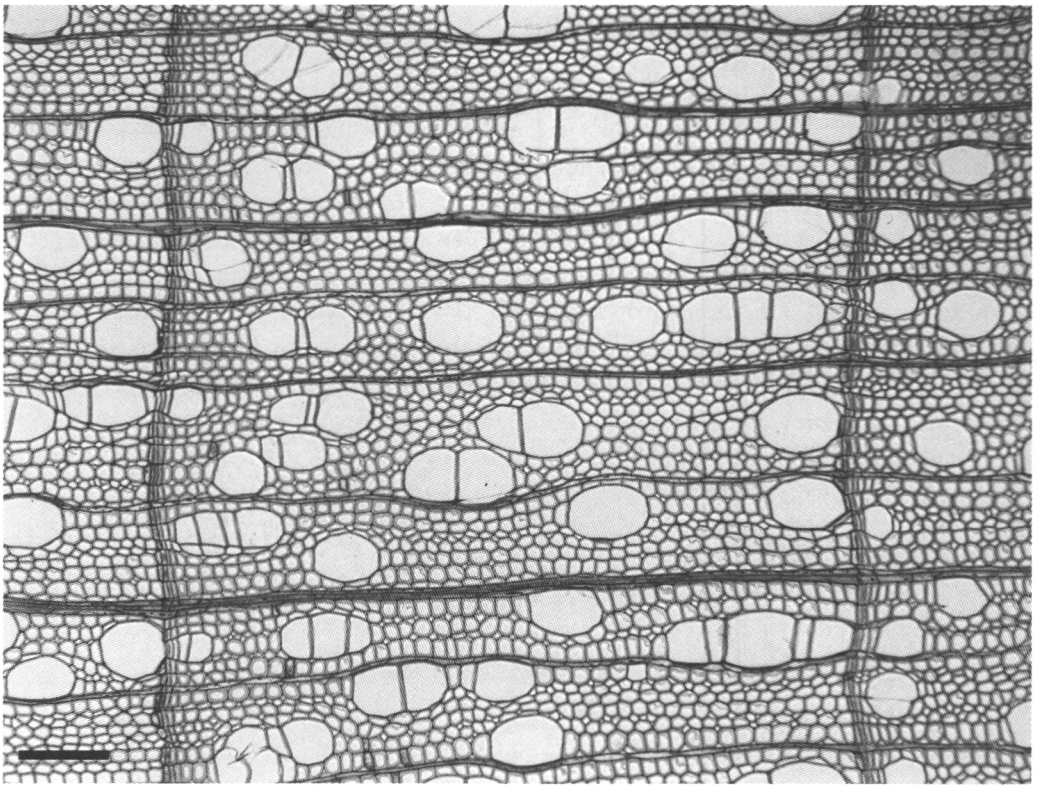

Figure 1. Transverse section of 33-year-old silver birch wood grown in southern Finland. Safranin-Alcian blue stain. The growth ring had a distance of 39 mm from the pith. Bar = 100 µm.

Silver birch is an economically important tree species, providing material for pulp, veneer, plywood and joinery to Finland, Scandinavia and Russia (Cameron 1996). Silver birch has a medium density when compared to other European hardwoods. The average basic density of Finnish silver birch wood material is 512 (± 42) kgm^{-3} (± SE) (Heräjärvi 2002). Birch wood generally is considered to be a highly valuable raw material for a range of wood products (Sachsse 1988, Verkasalo and Paukkonen 1999). In mechanical processing silver birch is mainly used for indoor products, because the tensile strength of birch wood is considered to be relatively low compared to the density of wood (Kärkkäinen 1984) and also because birch wood is vulnerable to attack by decay fungi, when exposed to humid conditions (Kucera and Myhra 1996).

1.2. The role of growth hormones (auxin and cytokinin) in wood formation and lignification

The major auxin in higher plants, indole acetic acid (IAA), is synthesised in the shoot apical tissues and actively transported towards the base of the plant (Jacobs 1952, Sachs

1981, Aloni 1995). An essential role for IAA in the initiation and growth of the vascular cambium is evident from experiments involving exogenous IAA (Little and Pharis 1995). The mechanisms controlling vascular differentiation during plant organ development and control of auxin biosynthesis have been intensively studied recently (e.g. Mattsson et al. 1999, Ljung et al. 2001, Schrader et al. 2003). Other aspects of auxin action in plant cells is the effect of auxin to stimulate elongation growth of plant organs, via a metabolically controlled increase in extensibility of primary walls (Taiz 1984), and the ability of auxin to control the orientation of microtubules, and thus, indirectly, of microfibrils deposited in the cell wall (Bergfeld et al. 1988). Leaf excision and IAA application studies have demonstrated that procambium development and primary xylem and phloem differentiation depend on a continuous supply of basipetally transported IAA (Jacobs and Morrow 1957, Bruck and Paolillo 1984, DeGroote and Larson 1984).

Dose-response experiments, where exogenously applied IAA has been used, have shown that e.g. in Scots pine (*Pinus sylvestris* L.) (Sundberg and Little 1990) an increase in the internal IAA level was positively related to increased tracheid production. In Scots pine this increase in IAA above a certain treshold value resulted in fewer tracheids (Sundberg and Little 1990). The reason for this was a low concentration of internal free IAA, rather than a high concentration of inhibitory level. Furthermore, a wide range of concentrations of exogenously applied IAA resulted in a relatively small range in internal IAA levels (Sundberg and Little 1990). The questions that have risen from the results of exogenously applied IAA dose-response experiments have lead to the development of other methods to study the effects of IAA on cambial division and wood formation. The idea that IAA regulates cambial cell division and development has been studied by determining the concentration of IAA (with the help of [$^{13}C_6$]IAA as an internal standard and GC-MS) in tangential sections taken through the cambial zone. This novel technique was first presented by Uggla and authors in 1996. The principal conclusion of the theory of IAA being a positional signal in pattern formation in plants is that the width of the radial concentration gradient of IAA regulates the radial number of dividing cells in the cambial meristem, which is an important component for determining cambial growth rate (Uggla et al. 1996, 1998).

Cytokinins form an other group of plant morphogens that can together with auxins and/or gibberellins stimulate cambial growth and early stages of vascular differentiation (Zakrzewski 1983, Minocha 1984, Aloni 1995). In the presence of auxin cytokinins influence the regeneration of vessels (Baum et al. 1991, Aloni 1995) and appear to promote fibre differentiation (Aloni 1982, Saks et al. 1984). However, cytokinins alone do not induce vascular differentiation in stem tissues (Aloni et al. 2000, Kijidani et al. 2001). The application of cytokinins results in similar responses to those elicited by defoliation or decapitation (Carmi and Koller 1978), which means reduced apical dominance, enhancement of cell devision, increased lateral bud burst and lateral stem elongation. There is evidence that roots are the main site of cytokinin biosynthesis (Short and Torrey 1972) and that transportation of cytokinins to the shoots of trees occurs in the xylem sap (Doumas and Zaerr 1988, Tromp and Ovaa 1990, Waseem et al. 1991) and that the endogenous

cytokinins or cytokinin-like compounds are located in the buds (Domanski and Kozlowski 1968, Hewitt and Wareing 1973, Qamaruddin et al. 1990). It has been found that in downy birch coppice shoots the amount of cytokinin-like compounds correlates positively with the xylem sap flow rate. The export (i.e. the amount of cytokinins transferred per unit time from roots to shoots) of cytokinin-like compounds, especially dihydrozeatinriboside- and zeatinriboside-types, was positively correlated with the initiation and elongation rate of downy birch coppice shoots, the number of lateral branches and the radial growth of the more slow growing coppice shoots (Rinne and Saarelainen 1994).

Numerous papers have reported that auxin-induced changes in peroxidase and IAA oxidase occur during rooting processes in tissue cultures (Mato et al. 1988, Fett-Neto et al. 1992, Liu et al. 1996). In indole-3-butyric acid (IBA) treated soybean (*Glycine max* (L.) Merrill) hypocotyls the endogenous IAA concentration increased and a decline in cationic and anionic peroxidase activities was detected (Chao et al. 2001). The decline in the anionic peroxidase activity (pI 3.5) was highly correlated with a decrease in the lignin contents in IBA-treated tissues (Chao et al. 2001). These results show that an increase in IAA content could have an effect on the lignin content in wood as well.

1.3. The impact of environmental and genetic factors on wood

1.3.1. Tension wood structure and formation

One-sided light conditions, uneven ground, snow load or wind cause one-sided longitudinal stresses to trees. Trees resist bending and the resulting repositioning of a stem is called reaction wood formation and occurs in single sides of many stems and branches. Thick stems need many years to reposition themselves even slightly (Archer and Wilson 1970, 1973). In broadleaved trees reaction wood is called tension wood, and it develops longitudinal stress several times greater than the growth stress in normal wood. In conifers the reaction wood is called compression wood that generates longitudinal compression in the opposite side in comparison to tension wood in broadleaved trees (Kubler 1987). Tension wood is usually found on the upper side of the leaning stem or of a branch and is often associated with eccentricity of growth that is to say wider growth rings being on the tension wood side (Dadswell and Wardrop 1955).

Tension wood does not differ macroscopically as much from normal wood as compression wood does. In silver birch and in downy birch (*Betula pubescens* Ehrh.) the surface of tension wood is seen as more pale, shiny and wax-like than normal wood surface (Ollinmaa 1955). The most characteristic macroscopic feature of tension wood is the extreme wooliness of saw-cut, when logs or boards containing tension wood are sawn longitudinally. The property is also seen in rotary peeled veneers. A possible reason for this feature is the lack of lignification in tension wood and the resulting abnormal bonding between individual cells (Dadswell and Wardrop 1955).

The well-known anatomical features of tension wood are fibres with a thick and highly refractive inner layer that is termed a gelatinous layer, a marked reduction in the size and

number of vessels and a higher average density (e.g. Ollinmaa 1955, Lassen 1959) than in normal wood (Dadswell and Wardrop 1955). Different types of tension wood fibres have been characterised according to their cell wall structure including several variations in the number of secondary wall layers, in micelle orientation and thickness and proportion of the innermost gelatinous layer (Dadswell and Wardrop 1955, Wardrop and Dadswell 1955). *Magnolia obovata* Thunb. and *M. kobus* DC. which are considered to be among primitive angiosperms, can form reaction wood without the typical gelatinous layer on the upper side of the inclined stem or branch. In addition, the size and number of vessels was reduced in reaction wood in these two *Magnolia* species (Yoshizawa et al. 2000).

Compression wood forms at sites, where auxin concentration is high. A high dose of applied IAA induces compression wood formation near the application site in conifers, whereas exogenous IAA suppresses tension wood formation in woody angiosperms (Little and Savidge 1987). In transgenic hybrid aspen (*Populus tremula* L. x *P. tremuloides* Minchx.) carrying a 35S-*rolC* gene construct a considerable amount of gelatinous fibres have been detected (Grünwald et al. 2001, the functions of *rol*-genes are explained in chapter 1.4. "*Agrobacterium*-mediated transformation"). It was concluded that the altered hormone levels may have directly stimulated the formation of tension wood in the transgenic hybrid aspens. However, the tension wood was also detected in control hybrid aspens and especially in plants grown in a greenhouse (Grünwald et al. 2001).

The best way to detect the presence of tension wood is by microscopic examination of thin cross-sections (15 to 18 µm). Its presence is usually revealed by the characteristic gelatinous inner layer of the fibres. If the normal fibres are especially thick-walled, the distinction is not clear, unless special staining is used e.g. safranin followed by light green. By this staining method not only whole bands of tension wood, but also isolated tension wood fibres can be detected, and the method is based on detecting the degree of lignification in the cell wall (Dadswell and Wardrop 1955). The light green stain or the zinc chloride-iodine treatment indicates the presence of a clearly defined gelatinous layer characteristic to tension wood (Dadswell and Wardrop 1955, Yoshizawa et al. 2000).

1.3.2. Changes in wood structure caused by different growth rates and by maturation

The properties of wood are a result of a long process, where genetic dispositions of the tree interact with environmental requirements (Kucera 1994). Environmental factors, such as fertilisation and thinning, have an effect on wood growth rate. Radial growth involves the division of meristematic cells and the differentiation of specialised cell types, and varies with such factors as genotype, age, within-tree position, site, weather and competition (Kozlowski et al. 1991). The interaction between wood properties and growth rate has been intensively studied in conifers (e.g. Saranpää 2003). A commonly presented hypothesis for softwoods is that the wider the growth ring is the lower the wood density and fibre length are (Dinwoodie 1965, Olesen 1976, Chalupka et al. 1977, Lindström 1996, Dutilleul et al. 1998, Mäkinen et al. 2002a, 2002b). In Norway spruce (*Picea abies* (L.) Karst.) trees a combined irrigation and fertilisation treatment resulted in increased growth rate and the volume of living cells (Stockfors and Linder 1998).

With a few exceptions, very little is known about cellular, molecular and developmental processes that underlie wood formation. Xylogenesis represents an example of cell differentiation in an exceptionally complex form and is controlled by a wide variety of factors both exogenous (photoperiod and temperature) and endogenous (phytohormones) and by interaction between the factors (Plomion et al. 2001). Recent findings have demonstrated the existence of an auxin (indole-3-acetic acid [IAA]) gradient across the developing vascular tissues of pine and poplar (Uggla et al. 1996, Tuominen et al. 1997, Uggla et al. 1998, Sundberg et al. 2000, Mellerowicz et al. 2001). This IAA concentration gradient seems to have a function in positional signaling, i.e. cambial derivatives develop according to their position along the gradient and neighboring cell files receive the same dose to develop in a synchronised manner. A sucrose gradient has been observed as well and may provide additional positional information for xylem and phloem differentiation (Uggla et al. 2001). Other hormones than auxins have shown to be involved in xylogenesis by interacting with IAA in a synergetic (gibberellins, cytokinins and ethylene) or inhibitory (abscisic acid) manner (Plomion et al. 2001).

When the diameter of trees increase and new growth rings are formed, the distance from the pith of the latest newly-formed xylem cells increases each year. During their development after cell division the xylem cells (fibres, vessels and tracheids) go through several processes, such as expansion, cell wall formation, programmed cell death, maturation to early- and latewood cells and finally, after several years, possible heartwood formation (Uggla et al. 2001, Plomion et al. 2001). The diameter of the stem and the distance from the pith e.g. the position of the cambial derivatives have an effect on the maturation process that is seen in the properties of the xylem cells, when mature xylem cells from different distances from the pith are compared and especially when juvenile and mature wood cells are compared (Larson 1994).

1.4. *Agrobacterium*-mediated transformation

Since the first successful transformation of a tree species in 1987 (Fillatti et al. 1987) transgenic trees have become essential tools for tree physiology research (Herschbach and Kopriva 2002). The first transformed tree species was a *Populus* hybrid (*Populus alba* x *grandidentata*) containing *Agrobacterium tumefaciens*-mediated *aro*A gene that was encoding 5-enolpyruvic acid-3-phosphate causing glyphosate herbicide resistency (Fillatti et al. 1987). Since plant hormones, (IAA, gibberellins, cytokinins and abscisic acid) in addition to the regulation of plant growth and development, also influence wood formation (Little and Savidge 1987), the manipulation of endogenous hormone levels in stem tissues of trees has been of great interest (Herschbach and Kopriva 2002).

A number of microorganisms that interact with plants use the same hormonal signals as plants. One of these microorganisms is *A. rhizogenes* that induces "hairy roots" (a proliferation of roots emerging from the wound site), from which whole plants with a characteristic phenotype including wrinkled leaves, shortened internodes and reduced apical dominance can sometimes regenerate (Gaudin et al. 1994). Roots induced by *A. rhizogenes* are branched and grow *in vitro* in the absence of growth factors and with modi-

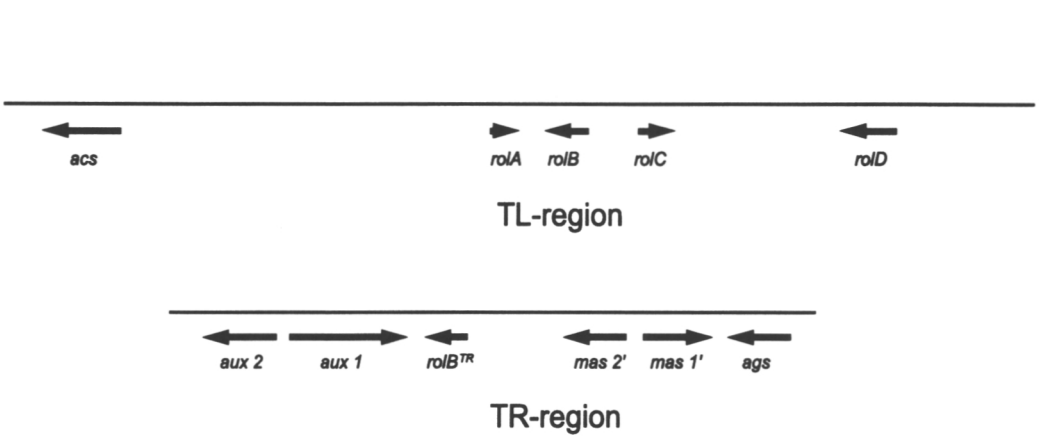

Figure 2. Structure of bipartite T-region of agropine *A. rhizogenes* strain A4 (redrawn from Gaudin et al. 1994). The TL-region contains the acs (agrocinopine synthesis), *rolA, rolB, rolC* and *rolD* (root loci) genes and the TR-region contains the *aux* (auxin biosynthesis), *mas* (mannopine synthesis), *ags* (agropine) and $rolB^{TR}$ genes (Gaudin et al. 1994)

fied geotropism (Gaudin et al. 1994). Hairy roots induced by *Agrobacterium* in plants result from the expression of certain genes passing from the bacteria to the plant. During the infection at a wound site, a part of a large plasmid (e.g. pRiA4 in *A. rhizogenes*, Figure 2), called T-DNA, is transferred from the bacteria and integrated stably into the plant genome. In spite of the prokaryotic origin, the gene expression is enabled in plant cells, because the T-DNA genes possess a eukaryotic type of regulation (Gaudin et al. 1994). The Ri plasmid of *A. rhizogenes* strain A4 shows an organisation with six oncogenes, the growth inducing genes (Figure 2).

Genes can be constitutive i.e. they are constantly transcribed, or they can be under tissue specific promoters or promoters that are activated at a certain developmental phase. The promoters are the key regulators that determine in which tissue and at which occasion the transferred gene is expressed. An often used promoter in transgenic tree studies is the cauliflower mosaic virus (CaMV) 35S promoter.

Several different plant species that have been regenerated from roots transformed by *A. rhizogenes* display developmental abnormalities such as formation of adventitious roots *in vitro*, altered root growth, wrinkled leaves, reduced apical dominance, shortened internodes or reduced pollen and seed production (Tepfer 1984, Gaudin et al. 1994). Genes *rolA, rolB, rolC* and *rolD* acting synergistically are responsible for this "*rol*" phenotype (Jouanin et al. 1987, Spena et al. 1987, Gaudin et al. 1994). The *rol* genes are associated with alterations in hormonal equilibrium, but their molecular function has not been completely explained.

Nilsson and Olsson (1997) have presented a model for the role of *rol* genes, especially for *rolB* and *rolC*. According to this they would act in concert to induce hair roots in the infection process, and *rolA* and *rolD* would further control and modulate this process. The

rolB protein is localised in the plasma membrane (Filippini et al. 1996) and stimulates root initiation in *Agrobacterium*-transformed tobacco leaf discs, when expressed under the 35S promoter (Spena et al. 1987). Two mechanisms of *rolB* action have been presented. The first theory hypothesised that *rolB* acts by increasing the pool of free, active auxin in transgenic plants (Estruch et al. 1991b). However, no differences between wild-type and *rolB*-expressing plants in the metabolic profiles of radiolabelled IAA or IAA conjugates were found (Nilsson et al. 1993). The second theory suggests that *rolB* is regulating sensitivity to IAA. *RolB*-transgenic tobacco protoplasts were 3000 to 100 000 times more sensitive to auxin than wild-type protoplasts, when the amount of NAA needed to induce transmembrane potential was measured (Maurel et al. 1994). The sensitivity of rolB protein to auxin is evident, but a controversy exists, while plants expressing *rolB* gene do not have a similar phenotype as auxin-overproducing plants expressing *iaaM* and *iaaH* genes (Nilsson et al. 1993b). The controversy was explained by the ability of plant cells to be responsive or nonresponsive to auxin (Nilsson and Olsson 1997).

The function of *RolC* protein to release free active cytokinins from their conjugates has been shown *in vitro* (Estruch et al. 1991a). When *rolC* is expressed under the strong 35S promoter the plants are dwarfed with very short internodes and an increased number of lanceolate light-green leaves. Their apical dominance is reduced, resulting in bushy plants (Schmülling et al. 1988, Nilsson et al. 1993a). Because of the antagonist effects of *rolB* and *rolC* genes and the bushy phenotype of *rolC* expressing plants it has been suggested that *rolC* expression may be coupled to an increase in cytokinin activity (Schmülling et al. 1988). Nilsson and Olsson (1997) suggested that *rolC* activity could positively influence cytokinin biosynthesis only locally and in young sink tissues.

The *tms* locus in pRiA4 has been shown to contain two genes encoding enzymes involved in a biosynthetic route for indole-3-acetic acid (IAA, Inzé et al. 1984). This IAA biosynthesis pathway does not exist in plants, and thus the corresponding genes and enzymes are probably not susceptible to plant regulation (Nilsson and Olsson 1997). The *aux1* gene (also known as gene *1, iaaM, tms1* and *shi*) encodes a tryptophan 2-monooxygenase catalyzing the conversion of L-tryptophan to indole-3-acetamide (Thomashow et al. 1986, van Onckelen et al. 1986), which is further modified to IAA by the *aux2* (also known as gene *2, iaaH, tms2* and *shi*) gene product, an indole-3-acetamide hydrolase (Schröder et al. 1984, Thomashow et al. 1984).

1.5. Wood formation and xylem properties in transgenic trees

The transgenic trees having changed growth properties have also been shown to contain a modified xylem structure. In transgenic hybrid aspen IAA overproduction was detected, when *iaaM* and *iaaH* genes under mas 1' and 2' promoters were expressed (Tuominen et al. 1995). These transgenic hybrid aspens had altered xylem formation, more uniform xylem with many intermediate-sized cells as a result of larger fibres and smaller vessels, when compared to normal wood in control plants (Tuominen et al. 1995). In addition the

cambial zone cells in transgenic hybrid aspens were more rounded and produced fibres that were more rounded than in control plants. The xylem in transgenic hybrid aspens was disorganised (Tuominen et al. 1995). These results show that by genetically modifying the IAA concentration in xylem, similar effects can be seen, e.g. IAA induced vessel density and size increase, as in experiments where auxin has been applied exogenously (Tuominen et al.1995).

The mechanism of auxin action in the cambial zone has been further studied in transgenic hybrid aspen containing *iaaM* and *iaaH* genes controlled by *mas* 2' and 1' promoters (Tuominen et al. 1997). In the cambium of transgenic trees the increased width and a lower peak level of the auxin gradient have been presented to be the primary causes for the longer duration of xylem expansion that leads to decreased xylem production and a larger lumen area of the produced fibres (Tuominen et al. 1997). The mechanisms of auxin action in transgenic trees have been studied by measuring the auxin levels in the tangential cell layers of the cambium. In addition to the absolute concentration of auxin in specific cell types controlling the rates of development in different tangential cell layers, the radial width of the auxin gradient determines the number of tangential cell layers being in the radial expansion phase. The transgenic trees exhibited an aberrant radial IAA gradient, which was shown to be related to altered xylem cell morphology (Tuominen et al. 1997).

The cell wall thickness and immunocytochemistry of lignin have been studied in the xylem of transgenic hybrid aspen having *rolC* gene under the 35S promoter (Grünwald et al. 2001). The wood of transgenic hybrid aspen had thinner fibre walls and less lignified secondary walls than the controls. More tension wood and gelatinous fibres were detected in the wood of transgenic hybrid aspens than in the control trees (Grünwald et al. 2001). The thinner walls, even though having less lignified secondary walls, usually indicate a higher total concentration of lignin because of the larger proportion of cell corners and middle lamellae, when compared to the total cell wall volume. However, the total concentration of lignin was not measured.

1.6. Defoliation as a stress factor and the mechanisms through which the effects of defoliation on tree growth are seen

Defoliation is an artificial procedure, where a certain amount of leaves or branches are removed from the plant. Artificial leaf removal is an environmental stress factor that mimics herbivory. By regulating the level of defoliation and the number of repeated defoliations the effects of herbivory as an environmental stress factor can be studied.

The way, the defoliation is done, has an effect on shoot elongation, cambial growth, phloem production and plant hormone concentrations (gibberellins, GAs, and indole-3-acetic acid, IAA, Wang et al. 1997). The mechanisms through which the defoliation affects plant growth lie on the basis of plant hormone physiology. It is a well known fact that

GAs play a major role in the mechanisms regulating shoot elongation and the production of xylem and phloem by the vascular cambium in woody species (Junttila 1991, Little and Pharis 1995, Moritz 1995, Olsen et al. 1995, Wang 1995a, 1995b, 1996). Moreover, indole-3-acetic acid is important for cambial growth in shoots of both conifers and woody angiosperms (Little and Pharis 1995). It is well documented that the activity of vascular cambium is inhibited when the supply of endogenous IAA to the cambial region is decreased by debudding, defoliation, girdling or by applying an inhibitor of basipetal IAA transport such as *N*-1-naphtylphtalamic acid (NPA), and is promoted by applying IAA to the apical cut surface of debudded shoots (Little and Savidge 1987, Little et al. 1990, Sundberg and Little 1990). In current-year shoots of Scots pine decapitation alone did not affect the concentrations of GAs, whereas defoliation reduced the concentrations of GAs (Wang et al. 1997). When two of the endogenous sources of IAA e.g. needle fascicles and shoot apex were removed, xylem and phloem production, longitudinal growth and stem IAA concentrations were decreased in the current-year shoots of Scots pine (Wang et al. 1997). The effects of decapitation alone were not so dramatic, because the supply of endogenous IAA from the needle fascicles was sufficient to maintain normal subapical meristem activity, although it was limiting for cambial growth (Wang et al. 1997).

Although the physiological mechanisms through which defoliation affects plants and artificial defoliation in plant-herbivore interactions have been studied intensively, the effects of defoliation on wood structure has been a rare subject for a study.

Changes in plants following damage or stress are called "induced responses" (Karban and Myers 1989). The consumption of foliage triggers induced responses in trees that may either decrease or increase the performance of individual insects (Haukioja 1990). When a plant is able to survive its first encounter with herbivores, it is usually able to respond to the consumption of leaves. Plant defences are assumed to develop as a result of herbivory or artificial defoliation and they are induced rather than a constitutive property in plants (Haukioja 1990).

Injuries to plant tissues cause a wide array of plant responses. The nature of the response varies with plant type. The way trees respond is associated with their growth pattern and nutrient status (Karban and Myers 1989). Secondary chemicals are known to affect plant resistance to herbivores. Many studies (e.g. Mutikainen et al. 2000) of induced responses have considered changes in tannins and phenols, products of the shikimic acid pathway. Relative activity of the enzyme phenylalanine ammonia lyase (PAL) can determine the production of phenolics, including lignin (DiCosmo and Towers 1984).

Compensatory ability after herbivory or artificial defoliation can be divided into three classes: overcompensation, equal compensation and undercompensation (Maschinski and Whitham 1989). When grazed plants are studied, the degree of compensation can be determined as the ability of the grazed plant to produce seeds and fruits. In overcompensation herbivory is beneficial for the plant and seed and fruit production exceeds that of the controls. In equal compensation the grazed plants produce equal numbers of seeds and fruits as controls and are not affected by the herbivores. In undercompensation herbivory is detrimental and the productivity of grazed plants is deteriorated in comparison to controls (Maschinski and Whitham 1989).

1.7. Storage and other objectives for extractives in wood

Storage is a common feature in perennial plants. Water soluble components such as carbohydrates including starch, soluble sugars and nitrogenous components including amino acids and proteins and water non-soluble components such as lipids (e.g. triacylglycerols) are stored in living tissues of woody perennial plants. The above mentioned storage compounds and also a wide variety of different secondary compounds that can be classified to several subgroups according to their chemical structure (mono- di- and triterpenoids, phenolic components, flavonoids, lignans, resin acids, etc.) are produced in the living wood cells and in coniferous trees in the epithelial cells of the resin ducts. The amount and composition of extractives is different, even in various parts of the same tree. Extractives, the secondary compounds, are intensively synthesised during heartwood formation (e.g. in Scots pine, Saranpää and Nyberg 1987a and 1987b, Saranpää and Höll 1989, Fischer and Höll 1992, Magel et al. 1994) heartwood being especially rich in secondary compounds.

Non-structural carbon compounds are stored in the wood mainly in axial and ray parenchyma cells. In the wood of silver birch storage fat (mainly triacylglycerol) is present in tiny fat droplets and starch in amyloplasts in living parenchyma cells (Harms and Sauter 1992).

The extractives do not only affect the (bio)chemistry of the trees, but also have an effect on the technological aspects of the wood such as wood quality and processing. The amount and composition of wood extractives effects the pulping and bleaching processes (Chen et al. 1995, Mustranta et al. 1995) and extractives may cause harmful discolouration during kiln drying (Mononen et al. 2002).

1.7.1. Seasonal variation in storage compounds

The most important task for storage compounds in woody tissues is to function as an energy reservoir, when the current demands for growth cannot be met by photosynthesis (Hansen and Grauslund 1973, Glerum and Balatinecz 1979, Kramer and Kozlowski 1979, Höll 1997). In addition, the non-structural carbon acts as a buffer for insufficient source activity (photosynthesis) due to adverse weather or loss of foliage (Li et al. 2002). Deciduous trees have been supposed to show more pronounced seasonal variation in non-structural carbon compounds than conifers (Kramer and Kozlowski 1979), because deciduous trees, unlike evergreens, are assumed to store large amounts of carbon reserves over winter in order to support stem and leaf growth after bud break and the beginning of radial growth (ring formation in ring-porous trees) during spring. As trees grow at their low temperature limit at this time it has been shown that the content of non-structural carbon (carbohydrates and lipids) in sapwood increases with elevation on both a dry matter and, even more so, on a volume basis (Hoch and Körner 2003, Körner 2003). Trees growing at the treeline show a changed source/sink relation. This results in a high abundance of non-structural carbohydrates and lipids, and they increase towards the low temperature limit (Körner 2003). The formation of new cells is much more sensitive to low temperatures (tempera-

tures close to zero) than photosynthesis (Körner 1999) and thus, the investment of carbon into structural growth is inhibited, and as a consequence of that the non-structural carbon pool increases (Körner 2003).

According to the predominant storage material in wood, tree species have been classified as "starch trees" and "fat trees" (Fischer 1891, Sinnot 1918). Based on this early classification, most ring-porous angiosperms, including some gymnosperms (e.g. species of *Abies* and *Picea*), belong to the group of "starch trees", while most diffuse-porous angiosperms (including the genus *Pinus*) are regarded as "fat trees".

1.7.2. Cold acclimation and membrane fluidity

Perennial plants, such as trees, depend on an efficient method to built-up frost resistance when growing in habitats with severe winter frost. The hardiness is induced during a relatively short period in the fall and is lost again in early spring (Sakai and Larcher 1987). There are several mechanisms including structural and biochemical changes in the living parenchyma cells of woody perennials that develop cold hardiness (Sauter et al. 1996). In boreal hardwood tree species the xylem ray parenchyma cells have been supposed to respond to subfreezing temperatures either by deep supercooling or by extracellular freezing (Sakai and Larcher 1987, Kuroda et al. 2003). Two types of chemical compounds, namely sucrose family oligosaccharides and free sterols, which are involved with cold acclimation processes and membrane fluidity in living wood cells, are dealt with in this thesis.

The important factors that have an effect on cold acclimation processes in the xylem of trees in the temperate and boreal zones are changes in total osmotic concentration of cell sap which are caused by changes in concentrations of sucrose and related oligosaccharides. The total concentration of non-structural carbohydrates, consisting mainly of sucrose, increases with hardening and decreases with dehardening of the cells (Sakai and Larcher 1987). In poplar (*Populus x canadensis* Moench "robusta") wood a rapid increase in the concentration of sucrose and its galactosides is concomitant to frost acclimatisation that starts during leaf fall (Sauter et al. 1996). Soluble sugars accumulate, when starch is hydrolysed to low-molecular weight carbohydrates (Sakai and Yoshida 1968, Levitt 1980). In parenchyma cells of poplar wood the activity of enzymes involved in sucrose metabolism, such as the sucrose-phosphate synthase (SPS), increased dramatically in the autumn concomitant to leaf fall, reached a maximum level in winter at the time of the starch-to-sugar conversion and declined in the spring during starch resynthesis and mobilisation (Schrader and Sauter 2002). During summer the activity of SPS remained at a very low level. The activity of sucrose synthase (SuSy) started to increase in late autumn, was high during winter and declined again in the spring (Schrader and Sauter 2002). In contrast to SPS, SuSy showed a remarkably high activity in the outermost parts of the stem of poplar (cells close to the cambium) in the summer, while it remained low in the middle and inner area of the trunk wood. This high SuSy activity was supposed to be associated with the differentiation of xylem cells (cell wall thickening) rather than with starch deposition (Schrader and Sauter 2002).

The total soluble sugar concentration correlates positively with cold hardiness. E.g. in poplar wood the concentration of sucrose and its galactosides (Sauter et al. 1996),

and in cortical and bud tissues of apple trees the concentration of sorbitol and raffinose (Stushnoff et al. 1993) correlated positively with cold hardiness. Sucrose and raffinose family oligosacchades are effective cryoprotectants. Sucrose interacts directly with the membrane bilayer. During dehydration stress, the hydroxyl groups of sucrose can replace water by hydrogen binding to the phospholipid head groups in the membrane bilayer, and they can prevent too close interaction between the head groups that would lead to gel phase transitions of the membrane bilayer (Anchordoguy et al. 1987, Crowe et al. 1987). Although sucrose is the principal agent in stabilising the membranes, small concentrations of raffinose family oligosaccharides may play a major role by preventing sucrose from crystallising (Koster and Leopold 1988).

Sterols are isoprenoid-derived lipids that have diverse and essential functions in all eukaryotes. Free sterols are integral components of the membrane bilayer, where, in conjugation with phospholipids, they regulate membrane permeability and fluidity (Clouse 2002). Plant membranes consist of a variable mixture of several sterols, sitosterol usually being the predominating sterol (Hartmann 1998). The changes that happen in membranes when the cells are stressed with low temperatures, have similar features as can be detected during senescense (Thompson 1984). Since campesterol is the most planar phytosterol, it may be expected to influence the physico-chemical properties of membranes in a similar way to that reported for cholesterol and is supposed to have adaptive value at chilling temperatures (Guye 1988). Furthermore, sitosterol is slightly more planar than stigmasterol, and it has been suggested that the maintenance of a high sitosterol to stigmasterol ratio would represent an adaptive chilling-tolerant response, while a decline in sitosterol to stigmasterol ratio would represent a non-adaptive chilling-sensitive response (Guye 1987 and 1988). In boreal tree species the sitosterol to stigmasterol ratio is usually very high especially in the xylem (e.g. in Scots pine, Saranpää and Nyberg 1987a), when compared to chilling-sensitive primary leaves of annual species such as bean (*Phaseolus vulgaris* L.) (Guye 1987).

2. The Aims of the Study

This is a study of silver birch wood and a study on the effects of environment and gene transfer and how they affect the structure and chemistry of wood. The basic aims of this study were to find out the reasons, why there is variation and what are the causes for the variation in the structure and the composition of secondary xylem. These primarily physiological events that happen in cambium, at the site where the cells divide and differentiate, lead to the changes in structure and composition that can be seen in the newly formed and older growth rings of xylem. The following means to study the effect of genes and environment on wood structure were chosen: transformation of *aux*- and *rol*- genes, defoliation, fertilisation and their combination. The changes in the concentration of storage components were followed during one season to study the wood as living tissue and to see the changes that happen in parenchyma cells during one season. The mature stems were studied to find out the within-stem variation in chemistry and the possible physiological reasons for that variation.

The main items can be presented by the following questions:

1) How transformation of principal plant growth hormone (IAA and cytokinins) biosynthesis-related genes affects wood structure and cell wall chemical composition?
2) What effects can be seen in wood structure after defoliation and fertilisation?
3) Can the changes in growth rate be seen in wood structure?
4) How does the extractive composition vary spatially within the stem?
5) Does silver birch form heartwood?
6) How should seasonal variation of extractives be studied in wood?

3. Material and Methods

3.1. Plant material

3.1.1. Silver birch clones

The clones in the defoliation and fertilisation experiments were micropropagated from commercially available saplings (Hortus-Puutarha Ltd., Kaarina, Finland), which were originated within latitudes from 61°08' to 63°18' N and within longitudes from 23°18' to 30°05' E (Mutikainen et al. 2000). The selection of these clones was based on their southern Finnish random origin. They also are commercially available clones that were cloned from a sample of trees that had been selected as phenotypically superior in terms of growth and quality and that had belonged to a breeding programme. One of the clones (no. 4 in II) used in the seasonal variation studies (III and IV), also named as V5952, was micropropagated by Stora Enso Ltd. in 1990 and originated from Valkeakoski, Finland (having coordinates 61°08' N, 28°49' E). This clone has been intensively studied by several authors and has been presented to manifest an overall resistency against mammal herbivores (Rousi et al. 1997, Tikkanen et al. 2003) and contains exceptionally high concentrations of secondary chemicals (defence-related secondary chemicals). However, this clone has not shown to be resistant against insects e.g. *Phytobia* fly (Ylioja et al. 2000).

3.1.2. Studies of structure, basic properties and cell wall chemistry

The transgenic and control trees were micropropagated (I) and the transformation was done using *Agrobacterium tumefaciens* – mediated technique (described in I) with a pRiA4 plasmid (Figure 2) as gene transferring vector. Before transformation the silver birch seedlings that were selected to *A. tumefaciens* inoculation were established with local (61°48'N; 29°17'E) seeds grown under normal greenhouse conditions (Aronen and Häggman 1995, I). The seedlings were grown in horticultural peat in containers 6 cm in diameter and were fertilised twice a month (with 0.1% - 0.2% commercial Superex fertiliser, Kekkilä) during the growing season, when the day and night temperatures in the greenhouse were kept above 15 and 10°C, respectively (Aronen and Häggman 1995). At the time of inoculation the seedlings were two months old (Aronen and Häggman 1995). The 5-year-old trees that were used to study wood anatomy and the 7-year-old trees that were used for the cell wall chemistry analysis were the original plants, which were confirmed to contain the *aux* and *rol* genes both at DNA level (by Southern analysis, I) and at RNA level (by Northern analysis, I). The most rigorously grown 7-year-old plants that were used to cell wall chemistry analysis and belonged to control group or to group IV containing all the *aux* and *rol* genes were decapitated due to greenhouse conditions.

The effects of five defoliation levels with or without fertilisation on silver birch wood properties and growth were studied in an incompletely randomised block design located in Suonenjoki, Finland (II). The fertility of the soil and other properties of the growth site are described in II. The experiment consisted of 15 clones. The study area contained of

ten blocks (9 x 24 m²). Five defoliation levels were conducted. None of the leaves or 25%, 50%, 75% and 100% of the leaves were removed by cutting the petioles with scissors. Buds were not removed and remained intact during the treatment. Each block had 60 groups containing four plants. Half of the randomly chosen groups (30) within each block was fertilised and the other half of the groups was not fertilised (Figure 1 in II). Each block contained two defoliation levels repeatedly (30 fertilised and 30 not fertilised groups). Within each block each clone had 16 representatives, which belonged to four groups each having different treatment (2 defoliation levels, with fertilisation and without fertilisation). Two of the plants belonging to each group were used in an earlier study (Mutikainen et al. 2000). The other of the two remaining plants belonging to each group were used in a biomass study (II). The last remaining plant of each group was used for a biomass study and a 10 cm long stem sample at the height of 40 cm was taken for this thesis (II). This experimental design resulted in four replicates (saplings) having similar treatment and being utilised for growth ring and basic property analysis. Altogether 600 saplings were included in the design of which 516 saplings were analysed for basic properties and growth. Four of the clones were randomly selected to study wood anatomy. The clones, as well as the treatments were randomly located within each block (Figure 1 in II). Altogether, 56 of the 64 planted saplings were analysed in wood anatomy studies.

3.1.3. Studies of variation in non-structural carbon compounds

The seasonal variation in non-structural carbon compounds was studied in a 7-year-old silver birch clone (III and IV). Using the clone as research material had several advantages; the selected trees were intact and the clone represented minimal genetic variation in growth properties (Table 1 in III). At six sampling times five randomly selected trees within the clone were felled (III, IV). The 7-year-old silver birch clone did not flower during the season of the study. According to Eriksson and Jonsson (1986) the female and male flowering starts in silver birch during the 7th and 8th growing season, respectively.

The mature trees to study the within-stem variation of non-structural carbon compounds were felled in Punkaharju in mid-summers during 1996 and 1997 (III, IV). The trees were located close to the growth site of young trees used in seasonal variation study and three of them were felled at the same time as five of the young trees during the rapid radial growth (III, IV).

3.2. Methods of wood anatomy and basic properties

The trees in both wood anatomy studies were five years old (I, II). The wood samples were boiled in water in a microwave oven for 1 - 4 min, frozen and thin transverse sections were cut at –14°C with a cryomicrotome (I, II). The sections were stained with safranin – alcian blue, rinsed with water, dehydrated in ascending alcohol series, rinsed with xylene and mounted in Canada balsam (I, II). The fibre and vessel length was measured from macerated samples taken from the outermost growth rings (I). The fibre, vessel and ray properties and transverse cell wall proportional area were measured with an Olympus

microscope connected to a video camera or Olympus microscope camera and using image analysis equipment (I, II). The image areas in vessel lumen diameter, vessel proportional area, cell wall index (CWI) and tangential ray area measurements were selected randomly representing the outermost growth ring from early to late wood (I) or the areas were taken 200 μm from the outer border of the growth ring representing the year of the treatment or either of the two growth rings formed after the treatment (II). The tension wood was avoided as much as possible in the area selection.

Growth ring width was determined with a computer aided system consisting of an Olympus stereo microscope connected to Heidenhein LS 303C transducer. The growth ring width was determined with an accuracy of 0.001 mm.

Wood density was measured with the water displacement method V^D (Olesen 1971). The green volume of silver birch disks was measured by detecting the apparent increase in green mass, when the disks were displaced in water. The green mass was measured before soaking the disks in water and the dry mass was measured after drying at 103°C for 48h (II).

3.3. Methods of wood chemistry

3.3.1. Cell wall chemistry

Lignin and carbohydrate concentration in 7-year-old silver birch saplings was measured from overnight at 70°C dried extractive free wood powder (I). Klason lignin concentration was determined after acid hydrolysis according to the gravimetric method of Effland (1977) and soluble lignin was measured by ultraviolet absorption at 203 nm from filtrate, using a lignin absorptivity of 110 l g^{-1} cm^{-1} (I). The filtrate, where meso-erythritol was added as internal standard, was neutralised with $BaCO_3$ and used for acid soluble carbohydrate measurements (Sundberg et al. 1996, I). Samples were silylated with N-trimethylsilylimidazole/pyridine (21:100, v/v; Brittain et al. 1971, I) and subjected to gas chromatography (I).

3.3.2. Non-structural carbon compounds

The total amount of acetone soluble extractives in mature trees is presented in this thesis only as a separate study. To determine the total amount of extractives in mature trees the wood was cut into match size pieces, dried overnight at 70°C and ground to a fine wood powder (Polymix mill, Kinematica). Two parallel samples of three grams of wood powder were extracted with acetone at 56.2°C for 6 h in a soxhlet apparatus. The amount of acetone soluble extractives was determined gravimetrically and the dry weight of the original wood powder was measured after drying at 103°C overnight.

For soluble sugar, starch, neutral and protein analysis frozen wood samples were cut into pieces of a half match size, lyophilised at –60°C and ground to a fine wood powder (Polymix mill, Kinematica, III, IV).

For soluble sugar analysis three parallel samples of wood powder were extracted with 80% ethanol (Mason and Slover 1971, III). Phenyl-ß-D-glucopyranoside was added

as an internal standard (Marcy and Carroll 1982, III). Samples were silylated with *N*-trimethylsilylimidazole/pyridine (21:100, v/v; Brittain et al. 1971, III) and subjected to gas chromatography (III).

For starch analysis the wood powder was heated at 100°C in a water bath to stop the enzyme activity of wood (Saranpää and Höll 1989, III). Three parallel extractions were made per wood sample. The starch of the samples was enzymatically hydrolysed (amyloglucosidase EC. 3.2.1.3; III). Phenyl-ß-D-glucopyranoside was used as an internal standard (Marcy and Carroll 1982, III). The samples were silylated with *N*-trimethylsilylimidazole/pyridine (21:100, v/v; Brittain et al. 1971, III) and subjected to gas chromatography (III).

For neutral lipid analysis the wood powder was extracted in a mini-Soxhlet apparatus with acetone and internal standards (triheptadecanoin, heptadecanoic acid, cholesterol and cholesteryl heptadecanoate) for 6 hours at 56.2°C (IV). The extracts were redissolved in diethyl ether and separated by thin-layer chromatography (IV). The fractions were located by spraying the edges of the plates with 0.001% primulin (Wright 1971, IV) and detected under UV-light. Corresponding untreated zones were scraped off and extracted four times with ethyl acetate (IV). The fractions (not free sterols) were saponified with 0.5 M KOH in 90% ethanol (IV) and acidified to pH 2.0 with 1 M HCl (Ekman 1979, IV). Fatty acid methyl esters (FAME) were prepared with boron trichloride methanol (IV) and free sterol and steryl ester fractions were silylated with water free pyridin, bis-trimethylsilyltrifluoroacetamide and trimethylchlorsilane (IV).

For protein analysis three parallel extractions in 0.05 M Tris-Maleate buffer, pH 7.7 containing 5 mM Na_2EDTA (Titriplex III), 1 mM $CaCl_2$, 10 mM $Na_2B_4O_7$, 1 M NaCl, 1.5% Polyclar AT and protease inhibitor were made (Fagerstedt et al. 1998, III). Protein contents of the extracts were determined with Bio-Rad microessay procedure (Bradford 1976, III).

The phospholipid thin layer chromatography (TLC) analysis is presented as a separate study in this thesis only. Three mature stems were felled in the 19th June, 2001 in Ruotsinkylä, southern Finland (66°96' N, 33°89' E), and one of the trees was chosen to analysis having a 32.5 cm diameter at breast height, a tree height of 26 m, a crown height of 11.6 m and an age of 75 years. Three samples (inner: 1-3 cm from the pith, middle: 3-6 cm from the pith and outer: 0-3 cm from the cambium) were taken at the height of 4.3 m and immediately frozen and stored at –80°C. The samples were cut into pieces of a half match size, lyophilised at –60°C and ground to a fine wood powder (Polymix mill, Kinematica) at –30°C. The samples of 500 mg were extracted with acetone in a soxhlet apparatus for 6 hours at 56.2°C and immediately dryed and diluted three times with 1 ml of chloroform:methanol (2:1, v/v, Bligh and Dyer 1959). Polyclar AT (20 mg) and 2 ml of ion-exchanged water (ISO 3696, water quality class 1) were added and samples were washed three times with 1 ml of Folch 2-phase (Folch et al. 1957). The samples were finally evaporated dry and diluted with 1 ml of chloroform:methanol (1:1, v/v).

Phospholipids were separated from the total lipid fraction by column chromatography (Unisil 100-120 mesh, Clarkson Chemical; Rouser et al. 1976). The eluant for neutral lipids was chloroform and acetone for glycolipids. Phospholipids were eluated with chloroform:methanol (1:1, v/v) and methanol and the neutral lipid and glycolipid fractions

were discarded (Piispanen and Saranpää 2002). The phospholipid fraction was further separated by TLC (Merck 1.05715, 0.25 mm silica gel 60 F_{254}). The plates were developed in chloroform:methanol:25% ammonia:water (115:45:3.75:3.75, v/v; Abramson and Blecher 1964) and stained with Sigma spray reagent molybdenum blue (M 3389) diluted 1:1 with 4.2 M sulfuric acid.

3.4. Statistical methods

Data were analysed by SPSS for Windows program (I, II, III, IV). The cell wall property data of transformed and non-transformed trees (I) and the seasonal variation data on non-structural carbon compounds (III, IV) were analysed by one-way ANOVA followed by the Student-Neuman-Keuls multiple range test and the Tukey test, respectively. The within-stem variation data on non-structural carbon compounds in mature trees were analysed by General Linear Model, which was composed of repeated measures ANOVA including Huynh-Feldt's test (acetone soluble extractives in separate study; III, IV).

Data on annual increment, vessel lumen diameter and vessel proportional area in defoliation and fertilisation experiment were analysed using a mixed model four-way ANOVA with year, defoliation and fertilisation as fixed effects and clone as a random effect (II). The annual increment in 1994 was used as covariate in the analysis of annual increment (II). Distance from the pith was used as covariate in the analysis of vessel lumen diameter and vessel proportional area (II). The relationship between vessel lumen diameter and distance of the sample from the pith was analysed with simple linear regression (II). Data on xylem water content and xylem basic density were analysed by three-way ANOVA with defoliation and fertilisation as fixed effects and clone as a random effect (II). Differences among treatments were analysed by pairwise comparisons with Bonferroni's test using the estimated marginal means of data to correct any discrepancies resulting from incomplete block design (Searle et al. 1980, II). The error terms were determined according to Zar (1984; II).

4. Results

In this thesis the results of substudies I, II, III and IV are summarised and drawn together. The detailed results of the separate studies are not repeated in the detailed form as they are in each paper (I, II, III, IV).

4.1. Wood anatomy and basic properties of wood affected by *aux*- and *rol*-genes, defoliation and fertilisation

Transformation with *rol* genes and 75% and 100% defoliation led to decreased growth ring width in five-year-old saplings when compared to control saplings of the same age under similar growth environments (Table 2 in I, Figures 2E, 2F and Table 1 in II). Fertilisation resulted in increased growth ring width at all defoliation levels (Figures 2E, 2F and Table 1 in II).

The *rolC* and *rolD* transformed trees had smaller vessel area and smaller vessel diameter than non-transformed control trees (Figures 5B, 5C in I, Figure 3A, 3B). In non-fertilised trees the 100% defoliation caused decreased vessel lumen diameter in relation to distance from the pith (Figure 6A and Table 5 in II) and when compared to the vessel diameter of control trees (Figure 3A). The plants that were grown in a greenhouse (transformed and non-transformed, I) contained tension wood, produced less xylem, had smaller vessel diameter and proportional area than plants grown in the field even when defoliated (I, II, Figure 3A, 3B). The *rol*-transformed plants had shorter vessels than control plants (Figure 6 in I). Fibres were also shorter in *rol* or both *rol* and *aux* gene transformed plants than in control plants (Figure 6 in I).

The concentration of structural carbohydrates (detected as total concentration of acid soluble carbohydrates) was smaller in *rolC* and *rolD* transformed plants than in control plants (Figure 7B, 7C in I). Total lignin content (Klason lignin + acid soluble lignin) showed an opposite trend between transformed and non-transformed plants when compared to structural carbohydrate concentrations (Figure 7 in I).

In 5-year-old silver birch trees the 75% defoliation lead to increased basic density of wood, and fertilisation resulted in decreased basic density of wood at all defoliation levels (II). Fertilisation caused increased moisture content of xylem when compared to control plants (II).

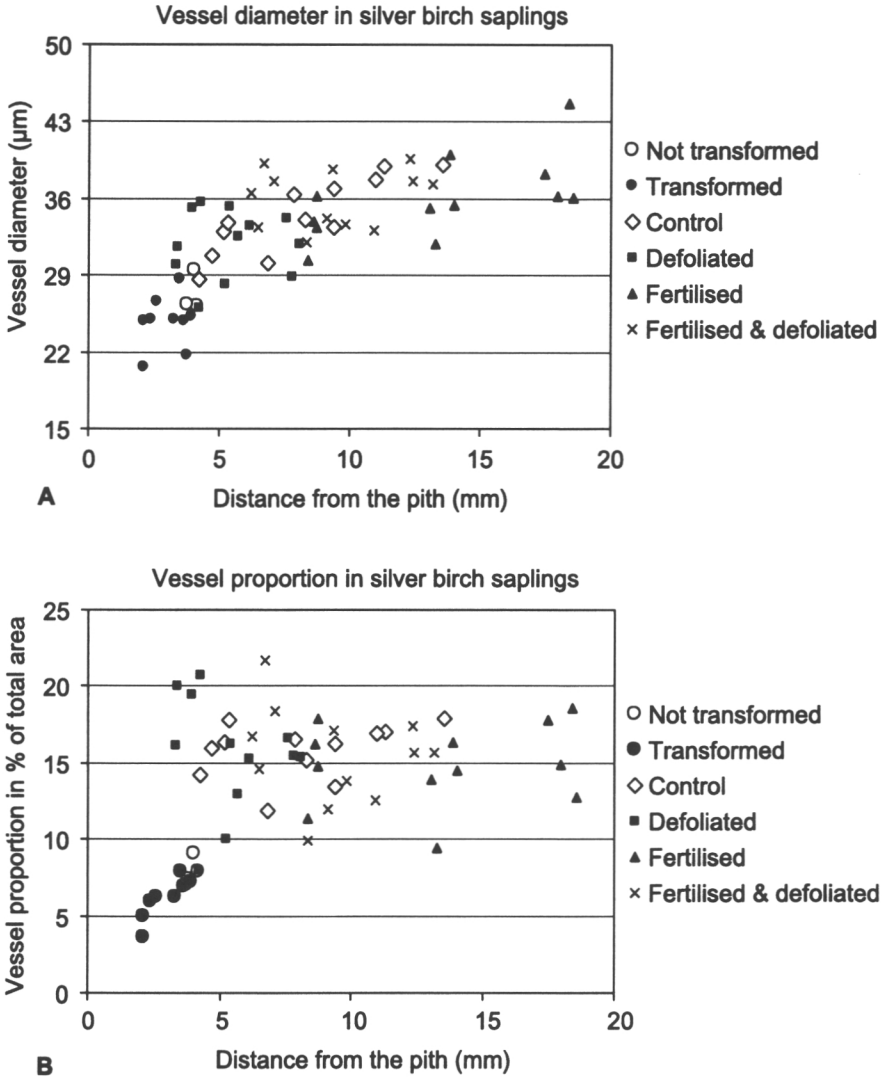

Figure 3. Vessel diameter (A) and vessel proportional area (B) in silver birch 5-year-old saplings. Each value is represented in relation to the sample's distance from the pith. Data collected from two separate studies (I, II). Transformed (●) and not transformed (○) plants in transformation study were grown in the greenhouse and each point represents the average vessel diameter (A) or proportion (B) in one individual plant. Control (◇), defoliated (■), fertilised (▲) and defoliated and fertilised (×) plants were grown in a field and each point represents the average vessel diameter (A) or proportion (B) of four individual plants belonging to same clone (II). Defoliation experiment data (◇, ■, ▲, ×) represent three separate growth rings. For statistics, see I and Table 5 in II.

4.2. Within-stem variation of non-structural carbon compounds

The results of total acetone soluble extractives are presented in this thesis only as a separate study. During rapid radial growth in the summer the average concentration of acetone soluble extractives in silver birch mature stems was 2.3 (± 0.2) % of wood dry mass (± SE). The concentration of extractives was significantly higher in the inner than in the outer parts of the wood (for repeated measures ANOVA, $F = 5.80$ and $P = 0.074$, Figure 4). At the height of 1 and 6 m the concentration of extractives was lower than at stump height (for repeated measures ANOVA, $F = 5.97$ and $P = 0.068$, Figure 4). The concentration of volatile compounds could not be analysed.

For the lipophilic compounds, like triacylglycerols, free fatty acids, free sterols and most of the esterified isoprenoids, the within-stem variation pattern in mature stems followed the general variation pattern of acetone soluble extractives (Figures 4 and 5 in IV, Figure 4). In contrast, the concentration of total soluble sugars, sucrose and glucose was highest in the outermost parts of mature stems, while starch and proteins did not show significant radial within-stem variation (Results and Figure 3 in III).

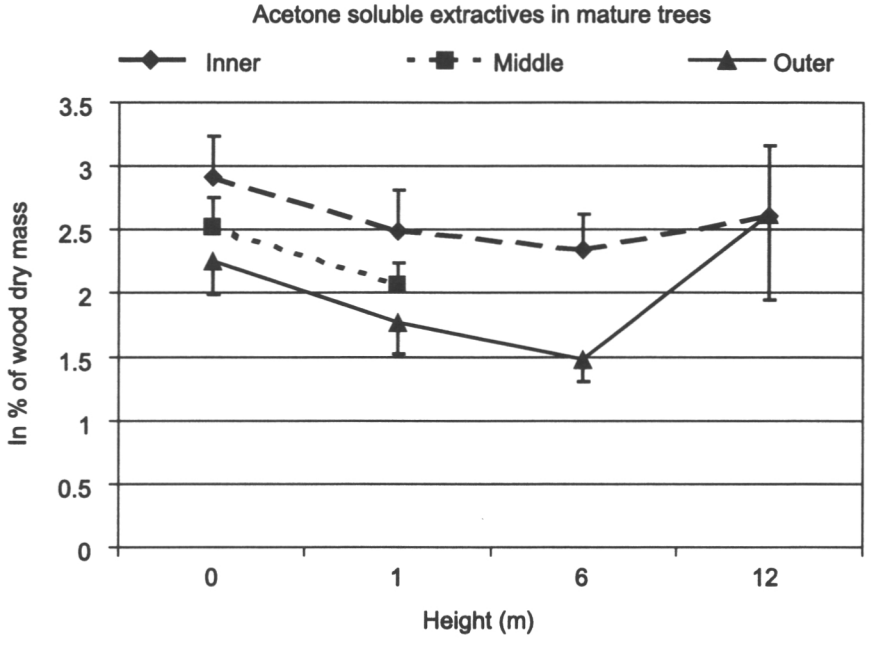

Figure 4. Partitioning of acetone soluble extractives within mature stems. Each line represents different distance from the pith: 1-3 cm from the pith (—◆—), 6-12 cm from the pith (- ■ -) and outermost sapwood, 1-3 cm from the cambium (—▲—). Each value connected with line represents the average extractive concentration of five independent trees expressed as % of extractive in wood dry mass. Error bars indicate tree-to-tree variation (standard error, n=5). Sampling procedure is presented in Figure 1 in IV. For sampling and statistical methods, see Methods in III and IV.

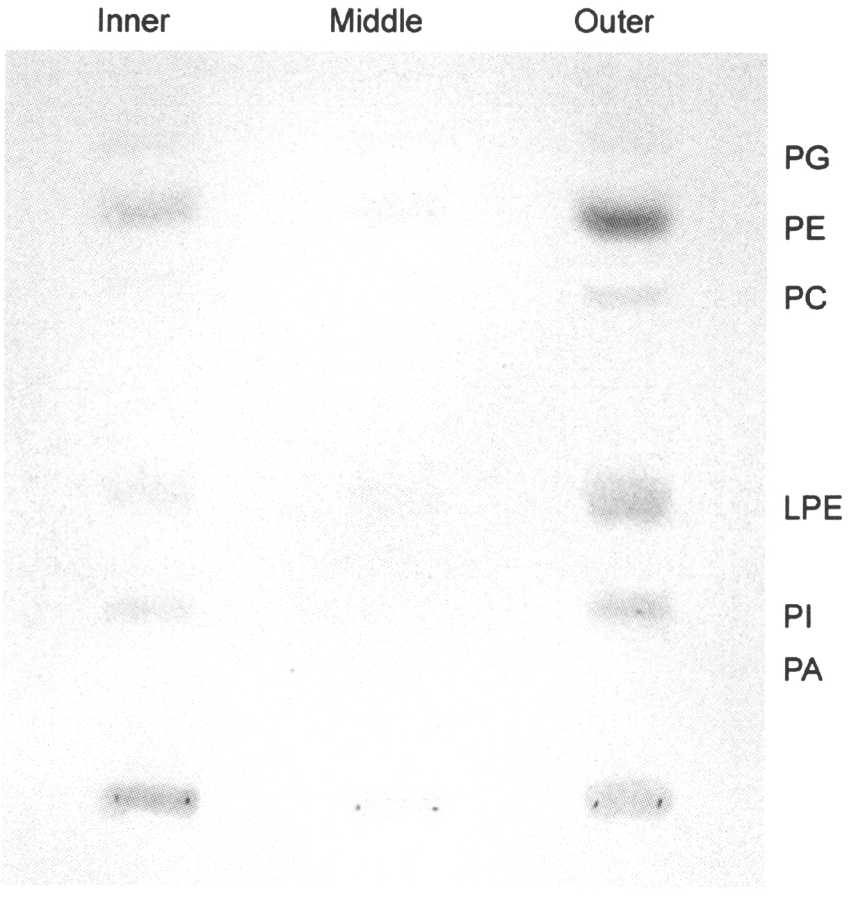

Figure 5. A thin-layer-chromatogram (TLC) of phospholipids from silver birch mature wood at the height of 4.3 m. Molybdenum stain. Each line represents samples from different distances from the pith (Inner: 1-3 cm from the pith, Middle: 3-6 cm from the pith, Outer: 0-3 cm from the cambium). PG = phosphatidylglycerol, PE = phosphatidylethanolamine, PC = phosphatidylcholine, LPE = lysophosphatidylethanolamine, PI = phosphatidylinositol and PA = phosphatidic acid.

The composition of phospholipid fraction is presented in this thesis only as a separate study. In mature silver birch wood lipid phosphorus from phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (LPE) was detected in samples taken from the inner, middle and outer parts of the wood (Figure 5). In addition, in the inner and outer parts of the wood lipid phosphorus from phosphatidylcholine (PC) and phosphatidylinositol (PI) was detected by qualitative analysis on TLC (Figure 5).

4.3. Seasonal variation of non-structural carbon compounds

The 7-year-old silver birch clone had lowest concentrations of non-structural carbohydrates in summer during rapid radial growth and highest, when the leaves were fallen

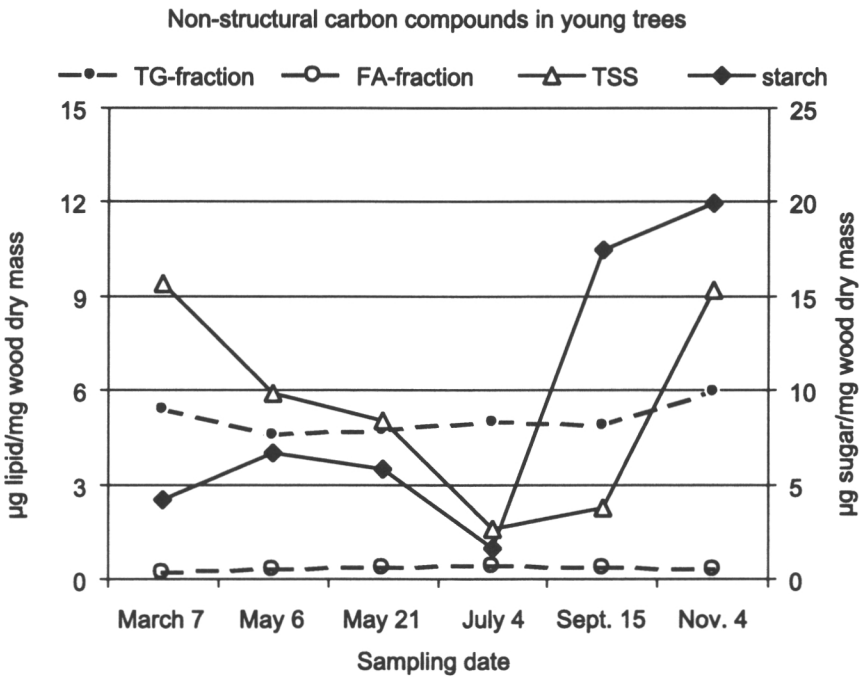

Figure 6. Seasonal variation in concentrations of storage compounds in 7-year-old silver birch stems. Each value connected with line represents the average concentration of five independent trees. Data collected from III and IV. Starch was measured as glucose units. TG fraction = triacylglycerol fatty acids, FA fraction = free fatty acids, TSS = total soluble sugars.

and the trees became dormant (Figure 2 in III, Figure 6), while in triacylglycerols only one fatty acid (palmitic acid in TG-fraction) showed similar seasonal variation (Figure 2 and Table 2 in IV, Figure 6).

Several compounds, like free fatty acid fraction (FA), free linoleic acid and esterified ß-sitosterol, cycloartenol, 24-methylenecycloartanol and campesterol had highest concentrations during rapid radial growth in summer (Figures 2A, 2B, 3A, 3B in IV). In contrast, the concentration and proportion of free ß-sitosterol decreased, when the temperature increased (Figure 3A in IV). In young silver birch wood ß-sitosterol showed obvious seasonal interconversion between free and esterified fractions and two betulaprenols (betulaprenol-7 and –8) also showed interconversion within the esterified fraction in their seasonal variation pattern (Figure 3 in IV).

5. Discussion

5.1. Wood anatomy and basic properties

In this thesis the results from two different types of experiments (transformation of growth hormone genes: I, and defoliation: II) were combined into one picture to show the importance of the within-stem spatial position, when cell size and proportion was studied. The comparison is relevant, because the trees in both studies were in juvenile phase and by drawing together these two completely different studies the influence of growth and maturation processes on wood properties is demonstrated. There is a slight connection between these two experiments; the consequences of the balance between the principal plant growth hormones, auxin and cytokinins and their role on wood formation are studied. Complete defoliation temporarily affects the hormonal balance of the plant (Savidge 1987, Little et al. 1990, Sundberg and Little 1990). Transformation of the principal plant growth hormone genes can have long term effects on the hormonal balance of the plant depending on the expression of the transferred genes and regulation of the hormone action (Christey 2001).

Defoliation and *rol*-transformation decreased the growth of silver birch saplings (I, II). This was seen in decreased production of xylem in defoliated and *rol*-transformed young trees, when compared to control trees (I, II). The rol-transformed trees and their respective control trees had smaller xylem diameter than defoliated trees and their respective controls (Figure 3). The difference in growth of the 5-year-old trees between these two experiments was caused by growth conditions and genetic origin. The plants were not suffering from nutrient depletion. The greenhouse-grown plants produced less xylem than the plants grown in the field (Figure 3). However, the plants in defoliation and fertilisation study (a field study) originated from commercial clones that had belonged to a breeding programme and had been selected to have phenotypically superior properties in terms of quality and growth. Some of these clones also had a more southern origin than the plants growing in the greenhouse that belonged to two clones that originated from seed material collected from Punkaharju, Finland.

The 5-year-old silver birch plants showed a distinct positive relation between vessel diameter and distance from the pith (Figure 3A). The relation between radial position and cell size is a typical phenomenon in wood and is especially emphasised in juvenile wood and has been detected in silver birch as well (Fabisiak and Helińska-Raczkowska 1997, Bonham and Barnett 2001). The differences in vessel size were consistent with their radial position pattern in juvenile silver birch stem. Irrespective of the treatment, e.g. transformation, defoliation or fertilisation, the vessel diameter seemed to increase in relation to the distance from the pith (Figure 3A). The effects of the treatments in vessel diameter of young silver birch trees were seen as secondary effects through the changes in the radial growth of the trees. It can be misleading to assess that the results are only caused by a treatment itself and not by a secondary effect of an increase or decrease in

growth. When very young trees are studied and when the studied trees are small in number, it can be difficult to show a distinct relation between the vessel diameter and distance from the pith. In the transformation study of silver birch a relation can be seen between vessel diameter and distance from the pith (Figure 3A) but was not statistically significant (I). In other studies made on transgenic poplar the plants were very small and the aspect of distance of the sample from the pith was not taken into consideration (Tuominen et al. 1995, Tuominen et al. 1997).

In young silver birch trees the radial pattern of vessel occurrence seemed to be more patchy than the radial pattern of vessel diameter (Figure 3). In silver birch wood it has been detected that the vessel proportion decreases from the pith outwards in the stem and branches (Bhat and Kärkkäinen 1981a) and also in mature wood (Kujala 1946, Ollinmaa 1955). The clones in the defoliation and fertilisation study seemed to follow the general pattern of vessel proportion detected in silver birch wood (Figure 6 in II, Figure 3B). The vessel occurrence and diameter were smaller in transformed trees and their respective greenhouse-grown control trees than in plants grown in the field (Figure 3). The vessel properties and growth of the plants in these two studies (I, II) were affected by environmental and genetic factors. The young trees in the transformation study had formed large amounts of tension wood irrespective of the treatment (I). In completely defoliated trees a zone of wood with thin-walled and incompletely lignified cells was detected in wood formed immediately after defoliation (in growth ring 1995), but no distinct tension wood formation was detected (II).

The *rol*-transformed trees showed an opposite behavior of vessel occurrence in relation to distance from the pith, when compared to the general trend in birch. In silver birch tension wood vessel occurrence is smaller than in normal wood (Ollinmaa 1955). When vessel proportional area was counted from young silver birch wood the tension wood was avoided as much as possible in the image area selection (I). The tension wood can have had an influence on the vessel proportion of trees in transformation study. The differences in vessel proportion between *rol*-transformed and control plants can be concluded to be a direct effect of the treatment (transformed *rolC* and *rolD* genes) and not as a secondary effect of growth. However, the fact that the trees were grown in the greenhouse and that they contained tension wood also have had an influence on wood structure.

The concentration of structural carbohydrates (detected as acid soluble carbohydrates) was lower in trees having *rol* genes than in the control trees (I). In the *rol*-transformed and control trees the lignin concentration showed an opposite but consistent trend when compared to concentrations of structural carbohydrates. It can be concluded that in the juvenile phase the *rolC* and *rolD* transformed silver birch trees showed consistent changes in vessel occurrence and concentrations of structural carbohydrates and lignin when compared to control trees. These changes were caused by the treatment and not only as secondary effects of growth. However, do these changes remain permanent after wood maturation, is an unsolved question.

5.2. Non-structural carbon compounds

The concentration of lipophilic extractives in silver birch wood showed an increase towards the pith and in longitudinal direction towards the stump height (Figures 4, 5 in IV, Figure 4). Soluble sugars, like sucrose and glucose showed an opposite behavior being most abundant in outermost parts of the wood (Figure 3 in III). Considerable between-tree variation in lipophilic extractives, like triacylglycerols, and in soluble sugars was detected especially above crown height (Figure 5B in IV, Figure 3 in III and Figure 4). To my knowledge, the vertical variation of extractives has not been studied in silver birch wood before. The samples from mature trees were collected in mid-summer, when the trees were in an active photosynthesising and growing phase. This can be seen especially in wood located above grown height in the amount and between-tree variation of sucrose, which is the transport form of carbon. In mature silver birch wood sucrose was concentrating on the outer parts of wood, on regions close to cambium and phloem, where the translocation of assimilated carbon is conducted. Thus, the pattern of sucrose partitioning in silver birch stem was physiologically sound.

Most of the lipophilic compounds, like triacylglycerols and isoprenoid compounds, concentrated close to pith in mature wood (Figure 4, 5 in IV). The horizontal variation in triacylglycerols followed the pattern previously found in silver birch (Höll and Poschenrieder 1975). Starch, proteins and lipid phosphorus were detected in mature wood close to the pith (III and Figure 5). The parenchyma cells close to the pith contained DNA in compact sickle-like structures (III). All these facts support the statement that silver birch does not form heartwood and that the parenchyma cells close to pith are living cells. The age of the mature silver birch trees used in this thesis varied from 30 to 75 years. The selected trees were intact and not infected by fungi that is to say they did not have a discoloured core wood typical to mature silver birches and they were superior and high-quality representatives of birch timber. Thus, they were optimal sample trees to study heartwood existence in silver birch.

In the heartwood-forming tree species, like Scots pine and black locust, the concentration of triacylglycerols decreases dramatically in the transition zone towards heartwood (Saranpää and Nyberg 1987a, Hillinger et al. 1996). In contrast phenolic extractives accumulate in the transition zone during heartwood formation (Magel et al. 1994). In silver birch a decrease in triacylglycerols or starch was not detected towards the pith (III, IV). Heartwood formation is a kind of programmed cell death in the inner parenchyma cells of wood that is not caused by pathological death or injury (Magel 2000). In this thesis the mature silver birch stems did not show any sign of heartwood formation according to the basic definition (Anonymous 1957). It was also shown that the parenchyma cells close to pith contained membraneous structures (Figure 5). However, to show thoroughly that the innermost parenchyma cells were living cells and protein synthesis existed in them would demand that RNA should have been detected in the cells.

In contrast to storage lipids, like triacylglycerols, soluble sugars and starch showed significant seasonal variation in young silver birch wood (Figure 6). The seasonal variation and a periodic reduction in the concentration of non-structural carbon compounds have

been contributed to indicate the tree's potential for carbon-driven stimulation of growth (Körner 2003). It has been suggested that the seasonal variation in non-structural carbon compounds would be more pronounced in deciduous trees than in conifers (Kramer and Kozlowski 1979), which was also stated in a recent study comprising several deciduous and coniferous tree species at a general level (Hoch et al. 2003). This thesis detected considerable variation in starch and soluble sugar pools of young silver birch trees during one season. The seasonal variation in non-structural carbon pools can be pronounced in very young trees. These silver birches were in juvenile phase and thus growing rapidly during summer and had a high photosynthetic need for carbon supply as well as high source and sink activity. In this thesis a 7-year-old clone was selected to minimise genetic variation between sample trees. In silver birch natural populations the secondary chemistry has been shown to be strongly affected by natural conditions, differences among genotype and their interactions (Laitinen et al. 2000). The response of individual trees to annual variation in the concentrations of secondary chemicals has shown to differ greatly between trees (Laitinen et al. 2000). The ideal experimental arrangement for a seasonal variation study is to use the same individual sample trees through the season. The use of a clone as experimental material to study seasonal variation was necessary in this case, because increment borings taken repeatedly from the same tree would have changed the physiology and also the carbon pools of silver birch.

In young silver birch wood the concentrations of sucrose and raffinose increased, when the temperature decreased (Figure 2A, 2C in III) and the ß-sitosterol/stigmasterol-ratio decreased towards mid-summer (Figure 3A in IV). Sucrose and its galactosides act as cryoprotectants in living cells (Koster and Leopold 1988). A high ß-sitosterol/stigmasterol-ratio indicates the plasma membranes' tolerance to low temperatures (Guye 1987, 1988). The changes in the concentrations of sucrose, raffinose and free ß-sitosterol can be connected to the changes that happen during frost hardening in membraneous structures in wood (III, IV). Interestingly, the changes in free ß-sitosterol concentrations could be coupled to variation pattern of the esterified form of ß-sitosterol (Figures 3A, 3B in IV). In this thesis a link between the basic physiology of the membraneous sterol compounds and secondary metabolism of esterified isoprenoids in silver birch wood could be found.

5.3. Conclusions

Silver birch, a deciduous boreal hardwood, has proven to be suitable material for transformation experiments. The transformation of *aux* and *rol* genes that affect principal plant growth hormone balance had an effect on wood structure and chemistry. These effects were direct and not solely caused by reduction of growth. The young birches in the transformation experiment were grown in a greenhouse, which also can have had an effect on wood properties (I). The transformed and control birches had formed large amounts of tension wood that might have had an influence on the results (I). However, the transformed birches showed a decrease in vessel size, vessel proportion and in concentration of structural carbohydrates (I). Of these changes only the decrease in vessel proportion

is a probable sign of increased amount of tension wood.

The defoliation and fertilisation field experiment, instead, is an example of how a treatment affects wood anatomical properties through growth. Defoliation decreased and fertilisation increased at every defoliation level the annual increment of birches (II). When vessel lumen diameter was plotted against distance of the sample from the pith, it was seen that irrespective of the treatment the vessel diameter increased towards cambium in young birches (Figure 3A). An opposite trend was detected, while vessel proportion decreased towards the cambium (Figure 3B).

The young birches in this study showed significant seasonal variation in the concentrations of starch, sucrose, glucose, fructose, raffinose, *myo*-inositol, free fatty acids, free linoleic acid, TG-bound palmitic acid, free ß-sitosterol, ß-sitostanol and campesterol, esterified ß-sitosterol, cycloartenol, citrostadienol, squalene and betulaprenol-7 (III, IV). The variation in these compounds was caused by changes in carbon allocation source-sink relations within the trees and biochemical changes that happen during cold acclimation and de-acclimation in wood. In mature stems lipophilic extractives, like triacylglycerols, TG-bound palmitic, linoleic and stearic acid, ß-sitosterol, ß-sitostanol, esterified campesterol, 24-methylenecycloartanol, squalene and betulaprenol-6, concentrated towards the pith (IV). Sucrose and glucose behaved in an opposite way and their concentration was highest in the outermost parts of the stems and also in wood located above crown height close to the carbon source and photosynthesising leaves (III). According to this thesis it can be stated that intact mature silver birch stems do not form heartwood (III, IV).

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Silver birch (*Betula pendula*) plants with *aux* and *rol* genes show consistent changes in morphology, xylem structure and chemistry

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Summary The effects of *Agrobacterium* pRiA4 *rol* and *aux* genes, controlled by their endogenous promoters, on tree growth and wood anatomy and chemistry were studied in 5- and 7-year-old silver birch (*Betula pendula* Roth) plants. Southern hybridization confirmed the following *rol* and *aux* gene combinations: control plants (no genes transferred); plants with *rolC* and *rolD* genes; plants with *rolA*, *rolB*, *rolC* and *rolD* genes; and plants with *rolA*, *rolB*, *rolC*, *rolD*, *aux1* and *aux2* genes. Transgene mRNA was most abundant in phloem/cambium samples and in the developing xylem, whereas no expression was detected in leaves. Plants with *rolC* and *rolD* genes or with all the *rol* genes were significantly shorter and had smaller leaves and a more bushy growth habit than control plants or plants with both *aux* and *rol* genes. Morphological observations and wood chemistry analyses revealed that plants with *rol* genes produced less xylem and broke bud later than control plants or plants with both *aux* and *rol* genes. Tension wood was detected in both control and transgenic plants irrespective of their gene combination, probably as a result of greenhouse cultivation. Xylem fibers were shorter in transgenic plants than in control plants, and plants with all the *rol* genes were characterized by shorter vessels compared with the control plants and a smaller proportional area of vessels compared with the other groups. In addition, silver birch plants with all the *rol* genes had approximately a 3.3% lower concentration of total acid soluble carbohydrates than control plants. We conclude that the *rolC* and *rolD* genes induced the typical "*rol*-phenotype," and that this was emphasized by concomitant expression of the *rolA* and *rolB* genes and alleviated by the presence of *aux1* and *aux2* genes. We observed consistent phenotypic effects of *rol* and *aux* genes on the morphology, anatomy and cell wall chemistry of the plants.

Keywords: anatomy, auxin, deciduous, transgenic, tree, wood.

Introduction

The chemical and physical properties of wood are important targets for tree breeding, and major research efforts involving molecular biology and genetic engineering are currently focused on lignification processes (Boerjan et al. 1997, Baucher et al. 1998). Plant hormones have an important role in regulating wood formation and structure (see reviews by Sundberg et al. (1997) and Herschbach and Kopriva (2002)). Indole-3-acetic acid (IAA), in particular, has been proposed as a key regulator that induces mitotic activity in the vascular cambium and xylem cell development, controls the integration of crown structure and stem form and serves as a link between external stimuli and wood formation. Manipulation of endogenous hormone concentrations in the stem tissues of trees by genetic modification can help to establish their specific roles.

A number of microorganisms that interact with plants carry genes that can modify hormonal balances or hormone signal perception in plant cells (Hamill 1993, Gaudin et al. 1994). Two principal types of oncogenes affecting auxin biosynthesis and sensitivity have been characterized in the pRiA4-plasmid of *Agrobacterium rhizogenes*. These oncogenes are *aux1* and *aux2*, which encode a novel pathway for IAA synthesis from tryptophan via indole-3-acetamide (IAM), and four loci involved in hairy root induction, called *rolA*, *rolB*, *rolC* and *rolD*. The *rol* genes are associated with alterations in hormonal equilibrium, but their molecular function has not been completely explained. Nilsson and Olsson (1997) suggested that *rolB* and *rolC*, regulated by sucrose and auxin, act in concert to induce hairy roots, and that *rolA* and *rolD* further control and modulate this process. The RolB protein, located on the plasma membrane and possessing tyrosine phosphatase activity, could increase auxin sensitivity of cells by enhancing their auxin-binding activity (Filippini et al. 1994, 1996). Alternatively, it has been suggested that RolB may hydrolyze inactive auxin-glucoside conjugates to the free, active form (Estruch et al. 1991). The RolC protein would then ensure a continuous supply of sugars to the growing meristem by interacting with sucrose transporters or by increasing local concentrations of cytokinins, thus creating a sink for assimilates, or by hydrolyz-

ing sucrose (Nilsson and Olsson 1997). Although RolA activity has been observed to result in a reduction in gibberellin concentrations and an increase in auxin sensitivity during the flowering stage (Gaudin et al. 1994, Nilsson and Olsson 1997), and *rolD* expression has been shown to induce early and enhanced flowering (Mauro et al. 1996), their exact functions are unknown.

The morphological and physiological effects of the pRiA4 oncogenes, i.e., the *aux* and *rol* genes, have been studied extensively in transgenic plants, using mostly isolated single genes under the control of their own promoter or a strong constitutive promoter such as CaMV 35S. Woody species, especially transgenic aspen (*Populus tremula* L.) and hybrid aspen (*Populus tremula* L. × *P. tremuloides* Minchx.) (Tuominen et al. 1995, 1997, Fladung et al. 1996, 1997*a*, 1997*b*, Nilsson et al. 1996*a*, 1996*b*, 1997, Tzfira et al. 1998), as well as kiwi (*Actinia deliciosa* A. chev.) (Rugini et al. 1991), *Solanum aviculare* Forst. (Jasik et al. 1997), *Rosa hybrida* L. (van der Salm et al. 1997) and apple rootstock (Holefors et al. 1998, Welander et al. 1998) have been used in these experiments. Common phenotypic alterations caused by expression of the T-DNA genes, especially *rolA*, *rolB* and *rolC* acting synergistically, include the formation of adventitious roots in vitro, altered root growth, wrinkled leaves, shortened internodes, reduced apical dominance, and inferior pollen or seed production. Wood characteristics such as the structure of individual xylem cells and the proportions and distribution of the xylem cell types are also modified in response to *aux* and *rol* gene expression (Tuominen et al. 1995, 1997, Nilsson et al. 1996*b*), thus demonstrating the possibility of manipulating wood properties through controlled changes in IAA concentration and distribution. Studies on the combined effects of *aux* and *rol* genes in transgenic trees have also been conducted, in which plants with either a normal or a hairy root phenotype have been regenerated following transformation by whole pRiA4 T-DNA (for a review, see Häggman and Aronen 2000), but these studies did not assess wood structure, anatomy or chemistry.

The objective of this study was to examine the effect of the pRiA4 genes *aux1*, *aux2*, *rolA*, *rolB*, *rolC* and *rolD*, controlled by their endogenous promoters on the growth, wood anatomy and wood chemistry of silver birch (*Betula pendula* Roth) and to compare the results with those of previous studies of single transgenes controlled by constitutive promoters. Simultaneous integration of these genes into transgenic plants allowed us to study interactions between transgenes.

Materials and methods

The Agrobacterium strain

The bacterial strain R1600, which has the chromosomal background of strain C58 (Wood et al. 2001) into which the Ri plasmid pRiA4b has been conjugated (Pythoud et al. 1987), was used for transformations. The bipartite T-DNA of the agropine-type pRiA4b plasmid consists of TL- and TR-regions. The TL-region contains the *acs* (agrocinopine synthesis), *rolA*, *rolB*, *rolC* and *rolD* (root loci) genes, and the TR-region contains the *aux* (auxin biosynthesis), *mas* (mannopine synthesis), *ags* (agropine synthesis) and $rolB^{TR}$ genes (reviewed by Gaudin et al. 1994). Strain R1600 also contains a pTVK291 plasmid (Komari et al. 1986) carrying copies of the *virA*, *virB*, *virG* and *virC* genes of pTiBO542, conferring a supervirulent phenotype (Pythoud et al. 1987). Strain R1600 was cultured in Luria Broth (Miller 1972) with 50 mg l^{-1} kanamycin.

Plant material

Two-month-old silver birch seedlings (n = 30) of local origin (Punkaharju, Finland: 61°48′ N, 29°17′ E), grown under normal greenhouse conditions as described by Aronen and Häggman (1995), were the target material for the bacterial inoculations. The experimental material was then derived from the hairy roots that appeared at the inoculation sites, as described below, and grown in a greenhouse under the same conditions as the original seedlings (see Aronen and Häggman 1995). The growing plants were transplanted to larger containers each year, and after four growing seasons the most vigorously growing plants (groups I and IV, see Results) were decapitated due to limited greenhouse space. Some of the experiments in which transgenic strains were characterized were performed on micropropagated material derived from these original greenhouse-grown plants.

Transformation protocol

Birch seedlings were transformed by inoculating wounded stems with a bacterial suspension pretreated with 100 µM acetosyringone in January 1992, as described in detail by Aronen and Häggman (1995). Two months later, a total of 16 hairy roots were excised from the inoculation sites, surface sterilized for 2 min in 70% ethanol and then for 20 min in 2% Ca-hypochlorite, rinsed with sterile water for 20 min and placed on phytohormone-free WPM medium (Lloyd and McCown 1980) solidified with 1% agar and containing 500 mg l^{-1} cefotaxime. One month later, the hairy root tissues were transferred to WPM medium containing 0.1 µM thidiazurone (TDZ) for shoot regeneration and after approximately 1 year of cultivation, a total of 162 shoots induced on six hairy roots were rooted on phytohormone-free WPM. They were transferred to a greenhouse in spring 1993.

Confirmation of transformation

The greenhouse-grown plants were tested as follows: genomic DNA was isolated from fresh leaves either for PCR analysis according to Doyle and Doyle (1990) with minor modifications described by Aronen and Häggman (1995), or for Southern hybridization according to the method of Lodhi et al. (1994) modified by Valjakka et al. (2000). For PCR screening, DNA samples were extracted from the plants during the first, second and fourth growing seasons. For Southern analysis, samples were prepared from fourth-year material.

Plants were screened with specific PCR primers (Table 1) to confirm the absence of agrobacteria in the regenerated material and to ascertain the presence of the *aux1*, *aux2*, *rolA*, *rolB*, *rolC* and *rolD* genes. In addition, the integrity of the template

Table 1. Specific pairs of primers used for PCR analysis of transformed material and preparation of double-stranded digoxigenin-11-dUTP-labeled probes for Southern and Northern hybridizations.

Primer sequences 5′-3′	Amplified fragment/probe	Analysis
CTGTCCTCAAATAGCGACCG CTGGCAACTGGTTGATTGCG	468-bp Fragment; nucleotides 112–581[1] of the trans-zeatin secretion (*tzs*) gene of the pRiA4 virulence region	PCR screening
CTCCGATTCCTTTCCAACCG CGCACGTTATCCTCATACCC	791-bp Fragment of *aux1*; nucleotides 3787–4578[2] from the pRiA4 TR-DNA	PCR screening Southern Northern
ATCGTGCATACTCACCACCG ACCTTCTTTGAGGTGTCGCC	516-bp Fragment of *aux1*; nucleotides 4918–5434[2] from the pRiA4 TR-DNA	Northern
CTGTCAACGGAGCTGTTGGG ACCCTAGTCTCATCCCAGGG	722-bp Fragment of *aux2*; nucleotides 1693–2415[2] from the pRiA4 TR-DNA	PCR screening Southern Northern
ACGGTGAGTGTGGTTGTAGG GCCACGTGCGTATTAATCCC	403-bp Fragment of *rolA*; nucleotides 9656–10,059[3] from the pRiA4 TL-DNA	PCR screening Southern Northern
TCAGGTTTACTGCAGCAGGC AACCTATTCGAGGGGATCCG	696-bp Fragment of *rolB*; nucleotides 10,520–11,216[3] from the pRiA4 TL-DNA	PCR screening Southern Northern
TGTGACAAGCAGCGATGAGC AAACTTGCACTCGCCATGCC	480-bp Fragment of *rolC*; nucleotides 12,513–12,993[3] from the pRiA4 TL-DNA	PCR screening Southern Northern
TGCCTTGAGGTCATTCATCAAGGCC ATGGACTGAAGGAGCACTCATTGGC	741-bp Fragment of *rolD*; nucleotides 16,634–17,375[3] from the pRiA4 TL-DNA	PCR screening Southern
CCTTACGAATTCTCTTAGCGGCACC GAGGTACACTGGACTGAATCTGCAC	477-bp Fragment of *rolD*, nucleotides 17,053–17,530[3] from the pRiA4 TL-DNA	Northern
CCTTCCACCACTTAGTTCCG TGATGCACTGGACTTGACGC	493-bp Fragment of birch ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*RbcS*) gene, nucleotides 210–703 of genomic clone	PCR screening

[1] According to Regier et al. (1989).
[2] According to Camilleri and Jouanin (1991).
[3] According to Slightom et al. (1986).

DNA was checked with primers specific to the birch endogenous gene. The reaction mixtures and conditions for PCR were as described by Aronen and Häggman (1995).

For Southern analysis, genomic DNA from the regenerated birch plants was digested with *Hin*dIII or *Eco*RI, after which DNA samples of 15–20 µg were electrophoresed on a 0.8% agarose gel and transferred to a nylon membrane (Boehringer Mannheim, Mannheim, Germany) by capillary transfer. The prehybridizations and hybridizations were performed in an Easy Hyb solution (Boehringer Mannheim) at 42 °C. Double-stranded probes for *aux1*, *aux2*, *rolA*, *rolB*, *rolC* and *rolD* were labeled with digoxigenin-11-dUTP (Boehringer Mannheim) by PCR using the same primers as for the preliminary screening (Table 1) but at an annealing temperature of 55 °C rather than 50 °C. After hybridization, membranes were washed twice for 5 min with 2× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) containing 0.1% SDS at room temperature and then twice for 15 min with 0.5× SSC containing 0.1% SDS at 68 °C. The digoxigenin-labeled hybridization products were detected according to the manufacturer's instructions.

Analysis of aux and rol gene function

Total RNA was extracted from samples of fresh leaves, phloem with cambium (phloem/cambium samples), and developing xylem (xylem samples) collected from 6-year-old greenhouse-grown plants during the growing season, according to the procedure of Chang et al. (1993). The total RNAs (15 µg) were separated on a denaturating 1.2% agarose gel and transferred to a nylon membrane by capillary transfer. Prehybridizations and hybridizations were performed in Easy Hyb solution at 50 °C. The digoxigenin-11-labeled double-stranded DNA probes for *aux1*, *aux2*, *rolA*, *rolB*, *rolC* and *rolD* are described in Table 1. After hybridization, membranes were washed twice for 5 min with 2× SSC containing 0.1% SDS at room temperature and then twice for 15 min with 0.2× SSC containing 0.1% SDS at 68 °C. The digoxigenin-labeled hybridization products were detected according to the manufacturer's instructions.

Micropropagation of regenerated plants

Plants originating from hairy root tissue (Clone 10) were prop-

agated in vitro from both dormant vegetative buds and twig internodes from 3-year-old greenhouse-grown plants as explants. Surface sterilization of the explants, tissue culture media and culture conditions were as described by Valjakka et al. (2000). The same protocols were used for plants derived from Clone 8, except that 0.1 μM TDZ, instead of 4.4 μM benzyladenine (BA), was used for induction of shoots on the bud explants, and 2.3 μM TDZ plus 0.03 μM 1-naphthaleneacetic acid (NAA), instead of 9.05 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.32 μM kinetin (KIN), were used for induction of callus on the internode explants.

Characterization of plants

Growth and morphology of regenerated plants in the greenhouse were monitored annually, and height, stem diameter (1–2 cm above ground), number of branches and leaf size, i.e., length of blade (four mature leaves from the top, central and basal parts of each plant), of 67 plants were measured during the fifth growing season (1997).

Root systems of micropropagated plants were studied in vitro. Randomly chosen individuals representing all *aux* and *rol* gene combinations, two individuals per group, were multiplied in vitro, and 20 shoots per clone were transferred onto phytohormone-free WPM medium, each shoot in its own culture jar. After a rooting period of 4 weeks, root systems were evaluated by counting the number of roots formed, visually assessing their hairiness and weighing the whole plants and excised roots after gently removing the tissue culture medium.

Wood anatomy

Three plants were selected from each transgenic and control group for anatomical studies 5 years after transformation. Sample disks taken at a height of 8 cm from the stem base were fixed in FAA (70% ethanol:acetic acid:40% formalin, 90:5:5, v/v/v) and the fixative was subsequently removed by boiling the samples in distilled water in a microwave oven for 1 to 4 min. The samples were frozen and 18 μm-thick transverse and 20 μm-thick tangential sections were cut at –14 °C with a Leitz 1516 cryo-microtome (Ernst Leitz, Ontario, Canada). Sections were stained with Safranin–Alcian blue (1% safranin O in 50% ethanol for 1 min followed by 1% alcian blue 8 GX in 0.05% acetic acid containing 0.04% formaldehyde), rinsed with water, dehydrated in an ascending alcohol series, rinsed with xylene and mounted in Canada balsam.

The cell wall index (CWI; cell wall area as a percent of total area in transverse sections), vessel lumen diameter and vessel area were measured on the transverse sections with the aid of an Olympus BX60 microscope (Olympus Optical, Tokyo, Japan) connected to a Panasonic WV–CD50 video camera (Matsushita Electric Industrial, Osaka, Japan) and the Image-Pro Plus 3.0 program for Windows (Media Cybernetics, Silver Spring, MD). Four randomly selected images of the outermost growth ring were analyzed per plant for the CWI measurements. One pixel corresponded to 0.51 μm, and the image area was 768 × 576 pixels. The CWI was calculated as area counts of bright objects subtracted from the total area counts in the image area; the dark areas of the image were deemed to be the cell walls of vessels, fibers, parenchyma rays and the axial parenchyma. For measurements of vessel lumen diameter and vessel proportional area, a maximum image area of 768 × 576 pixels was selected, excluding growth ring borders. One pixel corresponded to 1.06 μm. Three randomly selected images in the outermost growth ring were taken per plant and at least 77 vessels were measured per plant. Vessels that were only partly shown in the image area were deleted from the lumen diameter measurements but not from the vessel area measurements. The diameter was the mean of measurements made at 5° intervals around the centroid of the vessel lumen.

The ray parenchyma proportion in the tangential sections was measured from scanned color slides (Olympus BX-60 microscope, Olympus C-35AD-4 camera, EPY 64T Kodak film) with a Nikon Scan 2.2 (Tokyo, Japan) and the Image-Pro Plus 3.0 program for Windows. One pixel corresponded to 0.80 μm.

Two match-stick-sized pieces of wood were taken from the middle part of the outermost growth ring and macerated in glacial acetic acid/30% hydrogen peroxide (1:1, v/v) overnight at 60 °C for the vessel and fiber length measurements. One hundred fibers and 50 vessels per plant were measured with an Olympus BH-2 microscope and a CCDC Camera (COHU MOD 4912-5000/0000, Cohu, San Diego, CA) in conjuction with the Image-Pro Plus 3.0 program for Windows. One pixel corresponded to 2.70 μm and 0.11 μm in fiber length and vessel length measurements, respectively.

Lignin and acid-soluble carbohydrate measurements

Lignin and carbohydrate measurements were performed on four plants in each transgenic and control group 7 years after transformation. Wood samples (strips of length 20–30 cm) were taken at a height of 10 cm, debarked, dried overnight at 70 °C and ground to a fine powder (Polymix mill, Kinematica AG, Littau-Lucerne, Switzerland). Dry mass was determined at 103 °C. A sample of 3 g of wood powder was extracted with acetone in a soxhlet apparatus for 6 h; two parallel acetone extractions were performed per plant. The modified method of Effland (1977) was used for Klason lignin determination. Three parallel lignin and acid-soluble carbohydrate measurements were carried out per plant. A 300 mg sample of extractive-free wood powder was hydrolyzed in 1 ml of 72% sulfuric acid for 1 h in an ultrasonication bath, and about 82 ml of ion-exchanged water (ISO 3696, water quality class 1) and 2 ml (20 mg ml^{-1}) of meso-erythritol were added as an internal standard for the acid-soluble carbohydrate determinations. Sample solutions were autoclaved for 1 h (125 °C, 0.1 MPa) and filtered through fritted crucibles. After washing and drying, acid-insoluble Klason lignin was measured gravimetrically. The filtrate was diluted to 100 ml and acid-soluble lignin was measured by ultraviolet absorption at 203 nm with a Shimadzu spectrophotometer, using a lignin absorptivity of 110 l g^{-1} cm^{-1}.

A sample of 5 ml of the filtrate was neutralized with $BaCO_3$ for the determination of acid-soluble carbohydrates (Sundberg et al. 1996). Trimethylsilyl (TMS) derivatives of the carbohydrates were formed by evaporating neutralized samples (1 ml) to dryness and adding 400 μl of *N*-trimethylsilylimidazole/pyridine (21:100, v/v) (Brittain et al. 1971). After incubation

for 30 min at 80 °C, the TMS-derived carbohydrates were subjected to gas chromatography (GC) on a Hewlett Packard 5890 Series II gas chromatograph (Wilmington, DE) with a fire ionization detector and the HP GC ChemStation program (Agilent Technologies, Wilmington, DE).

Trimethylsilyl derivatives of acid-soluble carbohydrates were determined on a 25 m HP-5 (5% phenyl methyl siloxane, Hewlett Packard, Palo Alto, CA) column with an internal diameter of 0.2 mm and a film thickness of 0.33 µm. The column temperature was raised from 110 °C to 300 °C at a rate of 10 °C min^{-1}, and held at 300 °C for 16 min. Helium with an inlet pressure of 100 kPa served as the carrier gas. A split-injection mode was used: the split flow was 15 ml min^{-1} (split ratio 1:15), the septum purge was 3 ml min^{-1}, the injector volume was 1 µl and the injector temperature was 260 °C. Trimethylsilyl-carbohydrates were identified by co-chromatography of authentic TMS derivatives and by gas chromatography–mass spectrometry (HP 6890 GC system (Hewlett Packard, Waldbronn, Germany) with an HP 5873 mass selective detector at 70 eV (Hewlett Packard, Palo Alto, CA) and temperature program, GC column and gas flow adjustments as above).

Statistical analysis

Differences in growth, morphological and anatomical characteristics and the chemical composition of cell walls were examined by analysis of variance, and group means were compared using the Student-Newman-Keuls multiple range test.

Results

Regeneration of transgenic plants

Regeneration of plants from hairy roots was successful, but there was considerable variation in shoot formation ability of hairy root tissues on the WPM medium containing 0.1 µM TDZ. Although plants were derived from six hairy root clones, only Clones 8 and 10 produced numerous plants that grew vigorously under greenhouse conditions, and these were therefore selected for further studies. The absence of agrobacteria in these plants was confirmed by PCR screening, after which plants were divided into four groups (I–IV) according to their transgene combinations in the preliminary PCR analysis, as shown in Table 2. The PCR amplifications and preliminary results were confirmed when the plants were 1, 2 and 4 years old. Southern hybridizations (Figure 1) confirmed the insertion of bacterial *aux* and *rol* genes in plants in Groups I–IV, as expected based on the results of the PCR amplifications. Because the restriction enzymes cut the T-DNA at several points, the copy numbers of the inserted genes were not evaluated.

Expression of the transferred *rol* and *aux* genes was studied by Northern hybridization analyses of leaves, phloem with cambium, and developing xylem of plants from all groups (I, II, III and IV). No expression of the *rolA*, *rolB*, *rolC*, *aux1* or *aux2* genes was detected in leaf samples, but expression of the *rolD* gene was observed in the leaves of one plant in Group II that carried *rolC* and *rolD*. On the other hand, expression of all transgenes was observed in the phloem/cambium and xylem samples (Figure 2). Expression of *rolA* and *rolB* was observed in the phloem/cambium of the birch plants in Groups III and IV (carrying *rolA*, *rolB*, *rolC* and *rolD* without and with *aux1* and *aux2*, respectively), and a weak signal for *rolA* was also present in the xylem of the same plants. Genes *rolC* and *rolD* were expressed in both the phloem/cambium and xylem samples of plants in Groups II (carrying only *rolC* and *rolD*), III and IV, the *rolC* signals being stronger in the phloem/cam-

Table 2. Characteristics of 5-year-old silver birch plants with different combinations of bacterial *aux* and *rol* genes (Group I: no *aux* or *rol* genes; Group II: *rolC* and *rolD*; Group III: *rolA*, *rolB*, *rolC* and *rolD*; Group IV: *aux1*, *aux2*, *rolA*, *rolB*, *rolC* and *rolD*). Different letters within the same row indicate significant differences between groups of plants according to the Student-Newman-Keuls test ($P < 0.05$).

Characteristic	Group I[1]	Group II	Group III	Group IV[1]
Number of plants	31	7	6	23
Hairy root clone that plants originated from	10	10	10	8
Stem height (cm)	153 ± 9 a	88 ± 9 b	57 ± 7 b	140 ± 10 a
Stem diameter (mm)	12.1 ± 0.3	13.0 ± 0.9	12.5 ± 0.5	12.1 ± 0.5
Roots (percent of total fresh mass)	15.8 ± 1.4 a	28.6 ± 2.8 b	22.7 ± 1.7 b	16.4 ± 2.7 a
Leaf size (mm)				
Top leaves	73 ± 3 a	33 ± 2 b	35 ± 3 b	69 ± 3 a
Central leaves	49 ± 2 a	29 ± 3 b	28 ± 2 b	49 ± 2 a
Basal leaves	38 ± 1 a	24 ± 2 b	23 ± 2 b	37 ± 2 a
Percentage of plants with single stem	68	43	17	35
Xylem diameter[2] (mm)	8.5 ± 0.2 a	6.3 ± 0.5 bc	4.9 ± 0.6 c	7.6 ± 0.2 ab
Mean growth ring width[2] (mm)	0.98 ± 0.05 a	0.79 ± 0.07 b	0.56 ± 0.03 c	0.95 ± 0.02 a

[1] Plants were decapitated during the fourth growing season.
[2] Data from 12 individual plants.

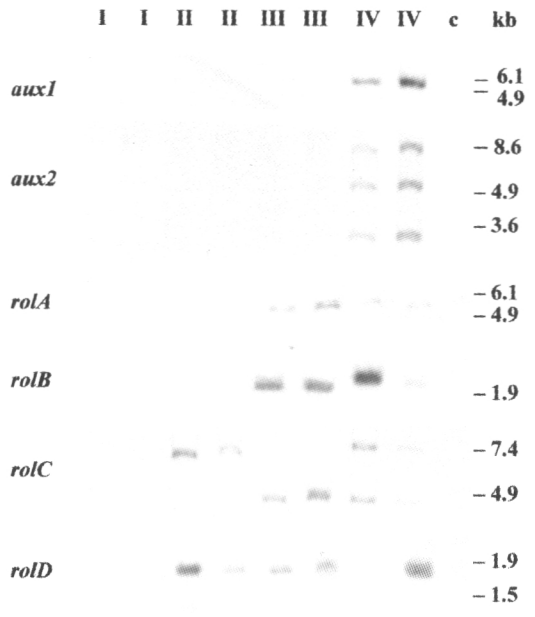

Figure 1. Southern analysis of silver birch plants regenerated from hairy roots. Genomic DNA (15–20 μg) digested with *Hin*dIII or *Eco*RI was loaded in each lane and analyzed with probes for *rolA*, *rolB*, *rolC*, *rolD*, *aux1* or *aux2*. The plants in Group I contained no transferred genes, whereas those in Group II contained *rolC* and *rolD*, those in Group III contained *rolA*, *rolB*, *rolC* and *rolD*, and those in Group IV contained *aux1*, *aux2*, *rolA*, *rolB*, *rolC* and *rolD*. The lane marked "c" was loaded with genomic DNA from a control birch plant, and the numbers on the right indicate the positions of the molecular weight markers.

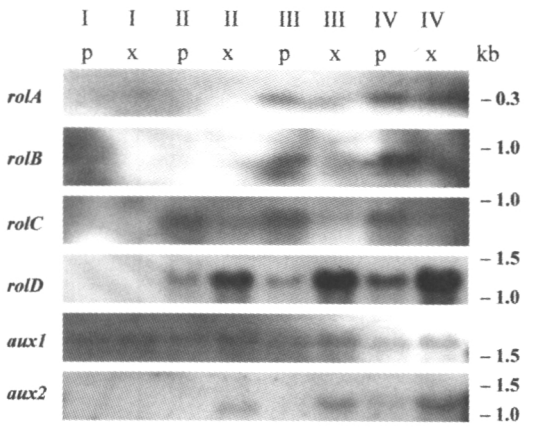

Figure 2. Northern analysis of silver birch plants in Groups I (no transferred genes), II (*rolC* and *rolD*), III (*rolA*, *rolB*, *rolC* and *rolD*) and IV (*aux1*, *aux2*, *rolA*, *rolB*, *rolC* and *rolD*). Each lane was loaded with 15 μg of total RNA; p = RNAs extracted from the phloem together with the cambium, and x = RNAs from developing xylem. The probes used are shown on the left, and the positions of the molecular weight markers on the right.

bium, and the *rolD* signals being stronger in the xylem (Figure 2). The *aux1* probes gave positive signals for all plants tested, indicating that they recognize not only the transgene but also an endogenous birch gene. With the *aux2* probe, signals were observed in both phloem/cambium samples and xylem of birch plants in Group IV, but were stronger in the xylem. There were also recognizable signals in the xylem samples from Groups II and III (Figure 2).

Characterization of plants

Morphological observations and growth measurements made in the greenhouse indicated marked variation in the characteristics of plants in Groups I–IV. As shown in Table 2, plants in Groups II and III (carrying only *rolC* and *rolD* or all the *rol* genes, respectively) were significantly shorter and had smaller leaves than plants in Groups I (no *aux* or *rol* genes) and IV (carrying both *aux* and *rol* genes). Their growth habit was bushy, they had slower bud break at the beginning of the growing season, and they produced less xylem than the controls (Figure 3, Table 2). The plants in Groups II and III also had significantly larger root systems in the in vitro experiment than those in Groups I and IV (Table 2) and their roots were more hairy, but there were no differences among groups in the numbers of roots formed.

Wood anatomy

Tension wood was detected in all transgenic and control silver birch plants (Figure 4). The amount of tension wood was not measured. The CWI (mean 52.0% ± 1.1 SE) and the mean proportion of tangential rays did not vary significantly between transgenic and control plants (Figure 5a), but the vessel proportional area and vessel diameter (mean 23.8 μm ± 1.3 SE) were lowest in Group III, although the result was significant only for vessel proportional area (Figures 5b and 5c). The mean lengths of vessels and fibers were 0.38 mm (± 0.02 SE) and 0.62 mm (± 0.02 SE), respectively, and the vessels were shorter in plants carrying only *rolC* and *rolD* (Group II) or all the *rol* genes (Group III) than in control plants (Group I) or those that also had *aux* genes (Group IV) ($F = 7.41$ and $P = 0.011$ in one-way ANOVA, Figure 6). Similarly, fibers were shorter in transgenic plants than in control plants ($F = 8.51$ and $P = 0.007$ in one-way ANOVA, Figure 6).

Lignin and acid-soluble carbohydrate measurements

Total lignin content (Klason lignin + acid-soluble lignin) of 6-year-old silver birch plants was approximately 20.4% (± 0.19%) of the wood dry mass (± SE). The Klason lignin and total lignin contents showed no significant variation between plant groups (Figure 7a).

Total acid-soluble carbohydrate concentration (arabinose + xylose + mannose + galactose + glucose) was lower in Group III plants, which carried all the *rol* genes, than in control plants or plants in Group IV, which carried all the *rol* genes as well as *aux1* and *aux2* ($F = 5.96$ and $P = 0.010$ in one-way ANOVA,

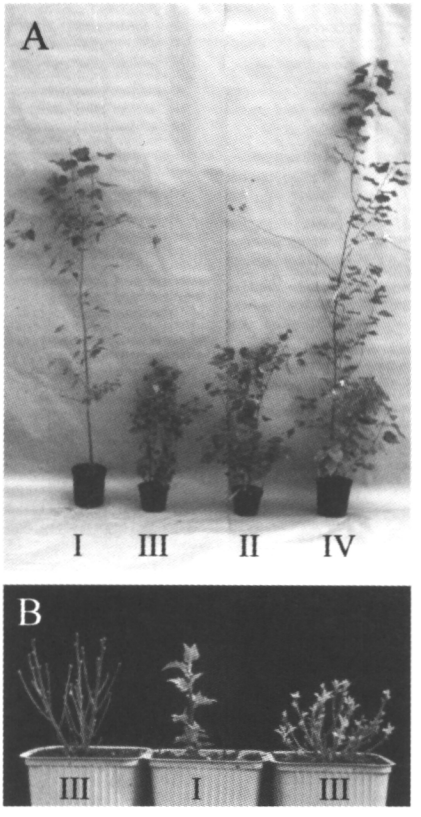

Figure 3. Transgenic silver birch plants in the greenhouse. Plants in Group I contained no transferred genes; those in Group II contained *rolC* and *rolD*; plants in Group III contained *rolA*, *rolB*, *rolC* and *rolD*; and those in Group IV contained *aux1*, *aux2*, *rolA*, *rolB*, *rolC* and *rolD*. (A) Plants regenerated directly from hairy root tissues in their fifth growing season. (B) Micropropagated plants at the beginning of their second growing season.

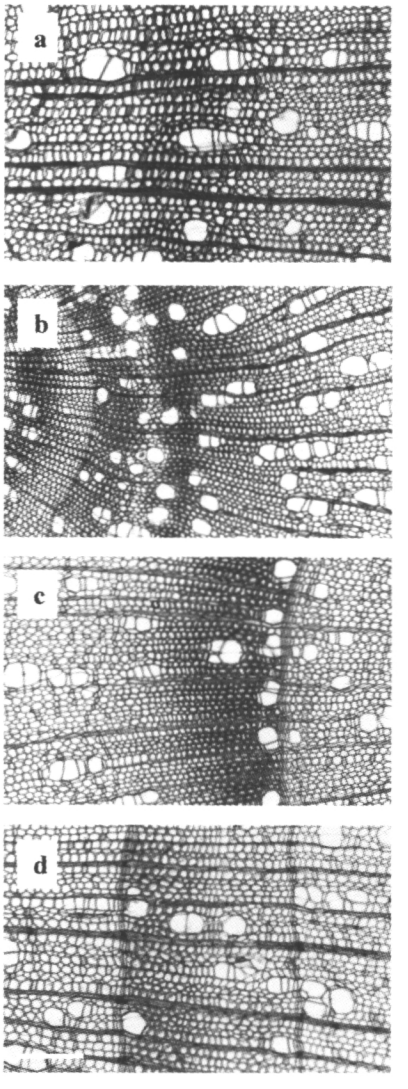

Figure 4. Transverse sections of silver birch xylem representing one plant from each of (a) Group I (no transferred genes), (b) Group II (*rolC* and *rolD*), (c) Group III (all the *rol* genes) and (d) Group IV (all the *rol* genes plus *aux1* and *aux2*). Tension wood is visible as a blue-stained, thick-walled area. Sections were stained with Safranine–Alcian blue. Bar = 100 µm.

Figure 7b). Glucose concentrations had the same distribution pattern as the total acid-soluble carbohydrate concentrations (Figure 7b), so that plants in Group III had the smallest glucose concentration ($F = 5.09$ and $P = 0.017$). Concentrations of arabinose ($F = 3.55$ and $P = 0.048$), mannose ($F = 5.30$ and $P = 0.015$) and xylose ($F = 18.42$ and $P = 0.000$) were slightly lower in transgenic than in control plants (Figures 7b and 7c), and the concentration of galactose was slightly higher in plants in Group IV than in the other transgenic plants or the control plants ($F = 3.17$ and $P = 0.064$, Figure 7c).

Discussion

Differences in morphology, anatomy and the chemical composition of the wood were observed between transgenic and control silver birch trees. Trees with different *rol* and *aux* gene combinations had different anatomical characteristics and wood chemistry. The transformation protocol that we used, i.e., regeneration of birch plants from hairy roots cultured in vitro, differs from the protocol generally employed, in which disarmed *Agrobacterium* strains are used. In other woody species, the use of oncogenic *A. rhizogenes* strains has resulted in regenerants with both normal (Han et al. 1993, 1997, Spiral et al. 1993, Tzfira et al. 1996) and hairy root phenotypes (Huang et al. 1991, Phelep et al. 1991, Devillard 1992, Shin et al. 1994,

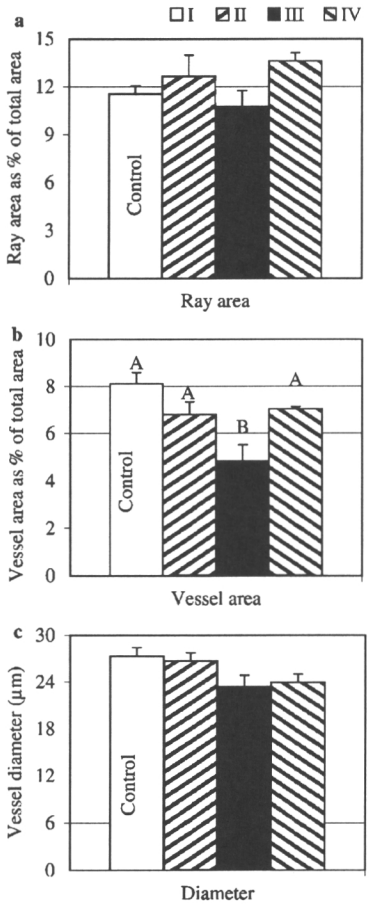

Figure 5. Ray proportional area (a), vessel proportional area (b) and vessel diameter (c) in tangential (a) and transverse sections (b, c) of control plants (I) and transgenic plants of Groups II (*rolC* and *rolD*), III (*rolA*, *rolB*, *rolC* and *rolD*) and IV (*rolA*, *rolB*, *rolC*, *rolD*, *aux1* and *aux2*). Different letters indicate significant differences between groups of plants (Student-Newman-Keuls' $P < 0.05$). Error bars indicate variation between plants (standard error, $n = 3$).

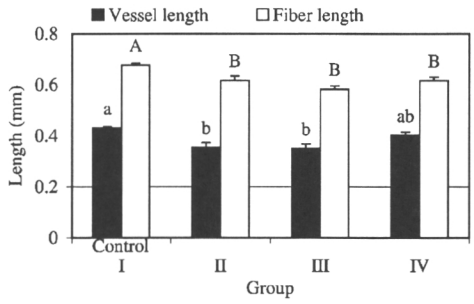

Figure 6. Vessel (solid bars) and fiber length (open bars) in control plants (I) and transgenic plants of Groups II (*rolC* and *rolD*), III (*rolA*, *rolB*, *rolC* and *rolD*) and IV (*rolA*, *rolB*, *rolC*, *rolD*, *aux1* and *aux2*). Different letters above similarly filled columns indicate significant differences between groups of plants (Student-Newman-Keuls' $P < 0.05$). Error bars indicate variation between plants within a group (standard error, $n = 3$).

Tzfira et al. 1996, Han et al. 1997), or in normal shoots with an extended root system (Lambert and Tepfer 1992). These reports, however, did not investigate the combination of pRi-oncogenes transferred or the function of these genes in different plant tissues.

Our studies on transcript abundance showed that the *rolA* and *rolB* transgenes under the control of their native promoters were expressed in the phloem/cambium samples from Groups III and IV, and that *rolA* was also expressed in the developing xylem of these plants (Figure 2). This observation is consistent with *rolA* and *rolB* expression patterns mainly confined to the phloem and root meristems (Nilsson and Olsson 1997). The *rolB* promoter has been shown to be activated by auxin (Maurel et al. 1990, 1994), and substantial amounts of IAA are known to be present in the phloem, although the maximum quantities are found in the vascular cambium (Nilsson and Olsson 1997).

The mRNA transcript levels of *rolC* in transgenic plant Groups II, III and IV were high in the phloem/cambium samples and low in the xylem during the rapid growth phase (Figure 2). In transgenic hybrid aspen plants, the *rolC* promoter was localized in living phloem cells when the current-year transgenic hybrid aspen shoots were growing rapidly in length and diameter (Nilsson et al. 1996*a*), and the promoter was later shown to function in the cambial meristem as well as its expanding derivatives (Tuominen et al. 2000). In addition, expression of the *rolC* promoter in the phloem has been observed in transgenic tobacco (*Nicotiana tabacum* L.) (Schmülling et al. 1989, Sugaya et al. 1989, Yokoyama et al. 1994) and rice (Matsuki et al. 1989). When growth declined in the transgenic hybrid aspen before dormancy, *rolC* promoter expression was also detected in the cortex and pith of current-year shoots, where carbohydrates are stored (Nilsson et al. 1996*a*). In transgenic hybrid aspen, the *rolC* promoter was activated by sucrose (Nilsson et al. 1996*a*). Because differentiating silver birch xylem contains living cells, e.g., parenchyma rays and axial parenchyma cells, it is also likely to express *rolC*.

The *rolD* gene under the control of its own promoter was most strongly expressed in the developing xylem of silver birch plants (Figure 2), at the point where the xylem element initials expand and differentiate into vessels and fibers. We also detected slightly weaker expression in the phloem/cambium (Figure 2). The *rolD* gene differs from the other *rol* genes in that it does not exhibit tissue-specific expression (Trovato et al. 1997). Instead, it seems to be developmentally regulated and is often expressed during the elongation, expansion and maturation phases of different tissues (Trovato et al. 1997). Thus the strongest expression of the *rolD* gene would be detected in developing silver birch xylem.

The various *aux1* probes used in the Northern analyses gave signals in the phloem/cambium and xylem of both transgenic

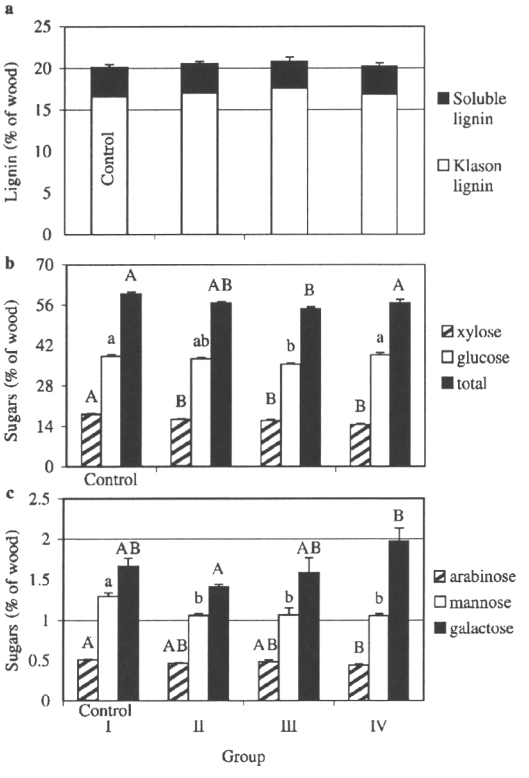

Figure 7. Concentrations of (a) lignin, (b) glucose, xylose, total acid-soluble carbohydrates, (c) arabinose, mannose and galactose in control plants of Group I and transgenic plants of Groups II (*rolC* and *rolD*), III (*rolA*, *rolB*, *rolC* and *rolD*) and IV (*aux1*, *aux2*, *rolA*, *rolB*, *rolC* and *rolD*). Error bars indicate variation in concentrations of total lignin (Klason + soluble lignin) and carbohydrates between plants (standard error, $n = 4$). Different letters above similarly filled columns indicate significant differences between groups of plants (Student-Newman-Keuls' $P < 0.05$).

and control birch plants (Figure 2), suggesting that silver birch plants produce an *aux1*-like mRNA that was recognized by the probes. Therefore, we cannot conclude whether the signal observed was due to expression of an endogenous gene or the transgene. The *aux2* signals were detected in the xylem and phloem/cambium of plants in Group IV (plants with all the *rol* and *aux* genes), but they were also observed in the xylem samples of Group II and III plants, which had no *aux* transgenes (Figure 2). One possible reason for our results is the expression of an *aux2*-like mRNA in the xylem of plants in Groups II and III, which could be activated by the action of *rolC* or *rolD*.

Morphology

Plants carrying only the *rolC* and *rolD* genes or all the *rol* genes had the shortest main stem and a bushy growth habit relative to the control plants (Figure 3 and Table 2). Annual *rolC*-transgenic plants, e.g., tobacco (Schmülling et al. 1988, Nilsson et al. 1993), potato (Fladung 1990) or *Atropa belladonna* L. (Kurioka et al. 1992), are reported to be dwarfed and to have an increased number of small, light-green leaves, short internodes and reduced apical dominance. Similar phenotypic changes were observed in the 5-year-old woody perennial silver birches with integrated *rol* genes in our study (Figure 3 and Table 2). These phenotypic alterations are caused by expression of the pRiA4 T-DNA genes, especially when *rolA*, *rolB* and *rolC* are acting synergistically (for a review, see Gaudin et al. 1994, Nilsson and Olsson 1997). The 35S-*rolC* gene construct in hybrid aspen resulted in a phenotype with drastically altered appearance, i.e., fasciated stems (Nilsson et al. 1996*b*, Sundberg et al. 1997), but no severely abnormal apical meristems producing flat stems growing in a spiral were detected in silver birch containing *rolC*, even though the growth habit of the transgenic birches with *rol* genes was stunted and bushy. The reason for the bushy but non-spiral growth habit of the transgenic silver birch trees could be low expression of *rolC*, which was insufficient to induce stem fasciation. In the hybrid aspen, a high level of expression near the axial meristems was needed to cause severe symptoms such as stem fasciation (Nilsson et al. 1996*b*). Expression of 35S-*rolC* was detected in leaves of transgenic hybrid aspen (Nilsson et al. 1996*b*), whereas expression of *rolC* under the control of its own promoter was not detected in the leaves of transgenic silver birch.

The polar flow of IAA maintains cambial activity and inhibits axillary bud outgrowth and leaf abscission (Cline 1991, Tuominen et al. 1995). The stronger outgrowth of axillary buds and branch formation in the transgenic silver birch trees in Groups II, III and IV than in control plants could indicate a lower concentration of active IAA or a smaller IAA to cytokinin ratio in the transgenics than in the control plants. The bushy silver birches (plants in Groups II and III) had not been decapitated.

The phenotypic changes observed in the birch trees correspond to the reported effects of *rolC* that mimic an increase in cytokinin activity (Nilsson and Olsson 1997), and it is possible that the influence of *rolD* may be stronger than has been previously assumed, as the phenotype of the birch trees expressing only *rolC* and *rolD* (Group II) did not differ much from that of plants carrying all *rol* genes (Group III). Expression of *rolD* is known to result in early, enhanced flowering in tobacco (Mauro et al. 1996), but the mode of action is unknown.

The finding that the silver birch plants in Groups II and III had smaller leaves than the controls or the Group IV plants is in accordance with observations of 35S-*rolC* transgenic hybrid aspen trees that had smaller, wrinkled leaves relative to non-transformed trees (Fladung et al. 1996, Nilsson 1996*b*, Grünwald et al. 2000, 2001). After a rooting period of 4 weeks, the proportion of total fresh mass contributed by roots was smaller in the control plants and in the Group IV plants than in those carrying only *rol* genes (Groups II and III; see Results). It has been found that explants from transgenic plants, e.g., kiwi (Rugini et al. 1991) and the woody perennial shrubs *S. aviculare* (Jasik et al. 1997) and *R. hybrida* (van der Salm et al. 1997) carrying *rolA*, *rolB* and *rolC* genes, showed an in-

creased ability to produce roots. In contrast, Grünwald et al. (2000) reported that 35S-*rolC* transgenic hybrid aspen trees had a smaller root volume than non-transformed control trees, but the ratio of root to total mass was not measured in that study (Grünwald et al. 2000).

Although obvious and significant reductions in height growth and xylem production were observed in plants in Groups II and III (Table 2), no reduction in stem diameter was observed, probably as a result of the slightly increased width of the bark or an increased number of knots in the transgenic plants. Reduced diameter growth has also been reported in 35S-*rolC*-transformed hybrid aspen trees relative to non-transformed trees (Grünwald et al. 2001). The reason that silver birch trees with *aux* genes (Group IV) are able to retain growth equivalent to that of control trees may lie in the function of either the transferred *aux1* and *aux2* genes or in the ability of endogenous birch genes to counterbalance the cytokinin-like effects of *rolC*, or in the fact that the plants in Group IV were regenerated from a different genotype (hairy root 8, Table 2).

Silver birch plants in our study contained a large amount of tension wood irrespective of the number of *rol* or *aux* genes transferred (Figure 4). The same phenomenon was observed in the 35S-*rolC* gene experiment in hybrid aspen trees grown in a greenhouse but not under field conditions (Grünwald et al. 2001). Growth environment also affects wood structure (Zobel and Jett 1995), and in this case probably caused tension wood formation. Formation of tension wood may have influenced the chemical composition and thickness of the cell wall. The amount of tension wood was not measured, but the phenomenon was evident in both the control and transgenic saplings.

Vessels and fibers

Fibers and vessels were shorter in transgenic plants (Groups II and III) than in control plants (Figure 6). In angiosperms with distinct growth rings, fiber length increases from the first-formed early wood to the last-formed late wood within one growth ring (Bisset and Dadswell 1950, Bisset et al. 1950). We took our samples from the same position within growth rings to ensure comparable measurements between plant groups. Indole-3-acetic acid is known to stimulate elongation in isolated segments of primary stem tissues (Cleland 1995, Napier and Venis 1995), and apically applied IAA is reported to have increased the length of xylem fibers in *Pyramimonas robusta* sp. Nov. (Digby and Wareing 1966). Tuominen et al. (1997), however, reported that the *iaaM* and *iaaH* (*aux1* and *aux2*) genes in transformed hybrid aspen trees had no influence on the length of xylem fibers, so it is evidently not the absolute amount of IAA that regulates the development of secondary xylem but rather its radial distribution pattern (Tuominen et al. 2000). In our experiments, the *rol* gene may have distorted the IAA balance in the birch trees of Groups II and III. That the vessels were shorter only in plants containing the *rol* gene is in accordance with the idea that the reduced apical dominance in Groups II and III is caused by changes in the ratio of IAA to cytokinin or by a reduction in the concentration of IAA.

The proportion of vessels was smaller in plants that contained all the *rol* genes (Group III) than in plants in the other groups (Figure 5b). In addition, vessel diameter was slightly less in plants with all the *rol* genes (Groups III and IV), although not significantly so (Figure 5c). Several studies of broad-leaved trees have shown that exogenously applied auxin increases vessel density and size up to a certain threshold, beyond which auxin inhibits vessel size but not density (Doley and Leyton 1968, Zakrzewski 1983, 1991, Meicenheimer and Larson 1985, Aloni 1991). These results support the idea that hormonal changes in the transgenic silver birch plants caused reductions in vessel and fiber size and vessel occurrence.

Cell wall chemistry

The concentration of total acid-soluble carbohydrates and glucose was lower in silver birch trees carrying the *rolA*, *rolB*, *rolC* and *rolD* genes than in the control trees (Figure 7b). Hu et al. (1999) have shown that a decrease in lignin content is compensated for by a simultaneous increase in cellulose content in transgenic aspen trees carrying a lignin biosynthetic pathway gene, which was down-regulated by antisense inhibition. Concentrations of cell wall structural elements in the transgenic silver birch plants in our study behaved in a consistent but opposite way, whereas the concentration of glucose was lower and the concentration of lignin slightly, but not significantly, higher in plants containing all the *rol* genes (Group III) than in plants of the other groups. It has been observed in hypocotyls of the soybean (*Glycine max* (L.) Merrill) during adventitious root formation that exogenous indole-3-butyric acid increased the endogenous IAA concentration and simultaneously reduced the lignin content (Chao et al. 2001). In our material, the slight increase in lignin content could have been caused by the *rol* genes, possibly through hormonal effects (reduction in IAA or a change in the IAA to cytokinin ratio). In the transgenic 35S-*rolC* hybrid aspen, the secondary walls of the extremely thin-walled fibers have been reported to be less lignified than control fibers, although UV-spectrophotometry revealed no distinct differences in lignification of xylem cells between transgenic and control hybrid aspens (Grünwald et al. 2001). In any case, the actual lignin concentration of the transgenic hybrid aspens was not measured.

Conclusions

Our results suggest that the *rolC* and *rolD* genes together can induce the typical "*rol* phenotype" in silver birch trees, including stunted bushy growth, smaller leaves and stronger root formation. This phenotype was further emphasized when the *rolA* and *rolB* genes were simultaneously expressed, thus supporting the *rol*-gene action motif hypothesis presented by Nilsson and Olsson (1997). The *rol* phenotype was alleviated when the *aux1* and *aux2* genes were co-expressed in the transgenic silver birch plants. Phenotypic effects of the *rol* and *aux* genes on the morphology, anatomy and cell wall chemistry of the plants were consistently observed.

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II

Effects of defoliation on growth, biomass allocation, and wood properties of *Betula pendula* clones grown at different nutrient levels

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Abstract: Three-year old *Betula pendula* Roth clones were grown at two nutrient levels in a field experiment to investigate the responses and recovery in growth and wood properties to a range of defoliation levels (0–100%). No general threshold value of defoliation level for negative effects in growth was found, since the sensitivity of saplings to defoliation varied according to plant traits studied. However, responses were related to defoliation intensity. Saplings compensated for 25% defoliation in terms of height growth and number of current branches and were able to tolerate 50% defoliation without effects on diameter growth 1 year after the defoliation. Nutrient availability was significant only in determining how total biomass responded to defoliation. Fertilized saplings were able to tolerate 25% defoliation without reduction in total biomass, but nonfertilized saplings were not. The interaction between defoliation and fertilization disappeared in the second growing season after the defoliation. Saplings were not able to compensate for 75% defoliation in terms of total biomass or for 100% defoliation in terms of growth and branching even in 2 years' recovery time. In stemwood, complete defoliation reduced growth ring width and vessel diameter simultaneously and also induced a narrow zone of secondary xylem with defects. Our results suggest that defoliation level and recovery time played a crucial role in compensatory growth of birch saplings, while nutrient availability had a minor role.

Résumé : Des clones de *Betula pendula* Roth âgés de 3 ans ont été cultivés en présence de deux niveaux de nutriments dans une expérience sur le terrain, pour étudier leurs réponses et la reprise de croissance ainsi que les propriétés du bois suite à une gamme d'intensité (0–100%) de défoliation. Aucun seuil général d'intensité de défoliation entraînant des effets négatifs sur la croissance n'a été observé étant donné que la sensibilité des gaules à la défoliation variait selon la caractéristique étudiée. Cependant, les réponses étaient reliées à l'intensité de la défoliation. Les gaules ont compensé une défoliation à 25% par la croissance en hauteur et le nombre de branches et pouvaient tolérer une défoliation à 50% sans que la croissance en diamètre soit affectée. La disponibilité de nutriments n'avait d'effet significatif que sur la réponse de la biomasse totale à la défoliation. Les gaules fertilisées pouvaient tolérer une défoliation à 25% sans réduction de la biomasse totale, ce qui n'était pas le cas des gaules non fertilisées. L'interaction entre la fertilisation et la défoliation est disparue au cours de la deuxième saison de croissance après la défoliation. Les gaules n'ont pu compenser une défoliation à 75% par la biomasse totale ni une défoliation à 100% par la croissance et la production de branches, même après 2 ans. Dans le bois de tronc, une défoliation complète a réduit simultanément la largeur du cerne et le diamètre des vaisseaux et a provoqué la formation d'une zone étroite de xylème secondaire avec des défauts. Le degré de défoliation et le temps nécessaire au recouvrement jouent un rôle plus crucial pour la croissance compensatoire des gaules de bouleau que la disponibilité des nutriments.

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Introduction

Although the effect of leaf-eating herbivores on plant performance may in some cases be highly detrimental, many plants are able to recover from foliar damage and compensate for the productivity of the leaf area lost (McNaughton 1979, 1983; Crawley 1983; Belsky 1986; Paige and Whitham 1987; Maschinski and Whitham 1989; Whitham et al. 1991; Reich et al. 1993; Trumble et al. 1993). The capacity of plants for compensatory growth after foliage damage is thought to depend on inherent plant growth rate and availability of light, water, and nutrients (Coley et al. 1985; Maschinski and Whitham 1989; Whitham et al. 1991).

The concept of a compensatory continuum (Maschinski and Whitham 1989; Whitham et al. 1991) suggests that plant

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responses to damage range from negative to positive depending, among other things, on resource availability and degree of the damage. For example, in previous work with silver birch (*Betula pendula* Roth), effects of partial (50%) defoliation on height and biomass were relatively stronger in fertilized saplings than in nonfertilized saplings (Mutikainen et al. 2000). Moreover, degree of compensation may depend on length of time available for recovery (Oesterheld and McNaughton 1991; Reich et al. 1993; Kaitaniemi et al. 1999). In most studies, defoliation has been simulated simply as a removal of 50 or 100% of leaf area whereas a range of defoliation levels has been rarely considered (but see Reich et al. 1993; Kaitaniemi et al. 1999). We know of no data about the interactive effects of a range of defoliation intensities, recovery time, and nutrient level on compensation in a single species.

Biotic and abiotic stress factors may reduce leaf area and lead to decrease in increment growth. Such reductions in increment growth have been reported, for example, in association with *Melampsora* leaf rust infection in poplar (Widin and Schipper 1981) and black cottonwood (Wang and Van Der Kamp 1992). However, few studies have considered defoliation effects on increment growth in birch species (Barter and Cameron 1955; Chalupa 1965; Hoogesteger and Karlsson 1992). It could be anticipated that reduction in increment growth affects stemwood properties, but at present, no such data are available to evaluate this hypothesis.

We used a field experiment to study how different defoliation intensities affect growth, biomass accumulation, and allocation, as well as xylem structure and properties (annual increment, basic density, moisture content, vessel diameter, and occurrence) of silver birch clones grown at two nutrient levels. The following questions were addressed: (*i*) are the effects of defoliation linearly related to defoliation level or is there a threshold level of defoliation for negative effects to occur; (*ii*) is recovery from defoliation different at high and at low nutrient level; and (*iii*) does length of recovery time affect the degree of recovery in silver birch saplings?

Materials and methods

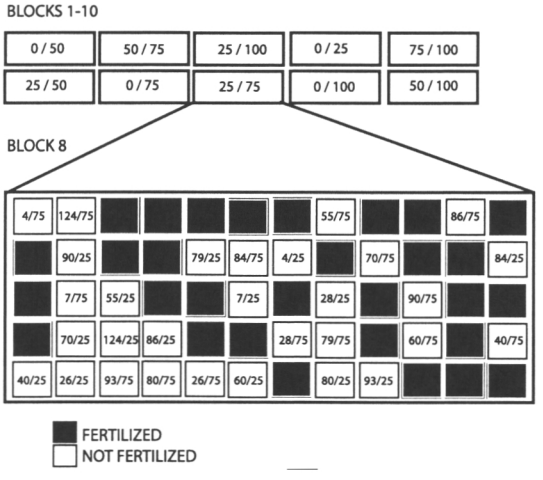
Plant material and experimental design

We used 15 micropropagated silver birch clones (Hortus-Puutarha Ltd., Kaarina, Finland) that originated from southern and central Finland. In May 1993, one-year-old saplings were transplanted to a field in Suonenjoki Research Station (62°39′N, 27°03′E, altitude 142 m a.s.l.) of the Finnish Forest Research Institute. At the time of defoliation, saplings were 3 years old.

The soil in study area is sandy with mean bulk density of 1.49 $g \cdot m^{-3}$ and contains, on average, 2.40% organic matter. The soil is composed of particles mainly in size classes between 0.06 and 2.0 mm with the highest proportion of particle size class 0.6–0.2 mm. The study area was divided in 10 blocks (Fig. 1). Altogether 240 saplings, i.e., 16 saplings from each of the 15 clones, were planted in each block (Mutikainen et al. 2000). Within a block, the 16 saplings from each clone were randomly divided to four subgroups each consisting of four plants, and there were 60 marked locations for the subgroups (Fig. 1). The location of each subgroup within a block was randomized. Two of these subgroups were fertilized (as described below), and two were not fertilized. Two plants of each subgroup were already harvested in experiments conducted in 1993 and 1994 (Mutikainen et al. 2000). A new defoliation experiment with the remaining two plants of each subgroup was conducted in 1995–1997 using randomized incomplete block design. Five different defoliation levels (0, 25, 50, 75, and 100% of leaves removed) and two fertilization (fertilized and nonfertilized) levels were studied. Each defoliation level had four replicates within clone. In this experimental design, each block had two defoliation levels, and each defoliation level occurred only once with another defoliation level within the same block (Fig. 1). Within each subgroup of the two saplings, one of the saplings was randomly assigned for measurements conducted in 1996 and the other for measurements to be conducted in 1997. From the total of 600 saplings in both years, six saplings had died in 1996 and 33 in 1997.

To achieve the desired defoliation levels one of four, every second, three of four, or all leaves were removed, respectively. The whole leaf lamina was removed by cutting the petiole with scissors. The saplings were defoliated between June 15 and July 13, 1995. The saplings were defoliated in a

Fig. 1. A diagram showing the experimental design. Two defoliation treatments were randomly assigned to each of the 10 blocks (upper panel, numbers within the blocks depict the defoliation treatments). Within each block, there were 4 groups from each of the 15 clones, 4 plants in each of the groups. The lower panel depicts a randomly chosen block (No. 8). The numbers in the lower panel refer to the clones and defoliation treatments (four groups for each clone). The position of each group within the block was randomly chosen and two randomly chosen groups of each clone were fertilized (solid squares) and two were not fertilized (open squares). Within each group, there were four plants. Two randomly chosen plants were used for previous experiments (1993 and 1994). For the experiment reported in the present paper, the other two plants within each group were defoliated in 1995 and their biomass were collected either in 1996 or 1997.



way to distribute any timing effects in the same way over each group to be analyzed as a unit.

The fertilized saplings had been treated using N–P–K fertilizer (18:5:10, Typpirikas Y-lannos, Kemira Ltd.) twice in 1993 and 1994 during the growing season. During the defoliation experiment, fertilizer was applied three times in 1995 (26 May, 8 June, and 13 July) and 1996 (10 June, 25 June, and 8 July), and four times in 1997 (14 May, 18 June, 3 July, and 17 July). In each fertilization event, 3 g of fertilizer was distributed around each of the saplings. By the end of 1997 the amount of fertilizer applied per sapling was 42 g. The amount of fertilizer used in 1993–1994 corresponded to a total of 12 kg $N \cdot ha^{-1} \cdot year^{-1}$, 3.3 kg $P \cdot ha^{-1} \cdot year^{-1}$, and 6.7 kg $K \cdot ha^{-1} \cdot year^{-1}$; in 1995–1996, it corresponded to a total of 18 kg $N \cdot ha^{-1} \cdot year^{-1}$, 4.9 kg $P \cdot ha^{-1} \cdot year^{-1}$, and 10 kg $K \cdot ha^{-1} \cdot year^{-1}$; and in 1997, it corresponded to a total of 24 kg $N \cdot ha^{-1} \cdot year^{-1}$, 6.6 kg $P \cdot ha^{-1} \cdot year^{-1}$, and 13.4 kg $K \cdot ha^{-1} \cdot year^{-1}$.

Growth, biomass allocation, and leaf properties

The growth and biomass measurements were carried out before the onset of autumn senescence, in the first half of August in 1996 and 1997. Height and diameter (middle of stem) of each sapling was measured and number of current branches was counted. Leaves, branches, and the stem of each sapling were collected and stored separately. The plant parts were oven-dried (80°C for at least 4 days until approximately constant mass) and weighed. In addition, in 1997, the area (measured using a LI-3000 leaf area meter, LI-COR, Inc., Lincoln, Nebr.) and dry mass of 10 randomly selected leaves from each sapling were measured and the total leaf area of each sapling was estimated using the following formula: (area of 10 leaves/dry mass of 10 leaves) × total leaf dry mass. The mean leaf size of each sapling was also calculated using the same leaves. Specific leaf area was then determined by dividing the total leaf area by total leaf dry mass of the sapling. Leaf area ratio (LAR) was further calculated by dividing the total leaf area by the total dry mass.

Wood properties

Pieces of stem at the height of 40 cm from the stump of the saplings were sampled for the analysis of wood properties in 1997.

Annual increment of the stems during years 1994, 1996, and 1997 was measured at the height of 40 cm. Total number of saplings analyzed was 516. Complete defoliation had severely damaged some of the saplings so that the terminal bud had died and an auxiliary bud had taken over the function of the main primary axis. Thus, the measurements were incomplete for growth ring width (see Table 1). Four centimetre thick disks (taken at the height of 40 cm) were analyzed for moisture content and basic density. Green volume of the disks was measured by the water displacement method (Olesen 1971). Dry mass of the sample disks was measured after drying at 103°C for 48 h. Moisture content was obtained by dividing green mass by ovendry mass of the disks.

Four of the 15 clones (clone Nos. 4, 26, 60, and 90; see Mutikainen et al. 2000) were randomly selected for detailed analysis of wood structure. Total number of saplings analyzed was 56. The vessel lumen diameter and the vessel area (as percentage in transverse section) were measured at the height of 40 cm in the three outermost growth rings of the control and 100% defoliated saplings. Narrow sectors of wood from pith to cambium or stem disks (stem diameter <1 cm) were boiled in water in a microwave oven for 1–4 min. The samples were frozen and 16 μm thick transverse sections were cut at –14°C with Leiz 1516 cryomicrotome. The sections were stained with safranin – alcian blue, rinsed with water, dehydrated in ascending alcohol series, rinsed with xylene, and mounted in Canada balsam (Merck 1.01691). The vessel lumen diameter and vessel area were measured using Olympus BX60 microscope connected to Panasonic WV-CD50 video camera and Image-Pro plus 1.0 for Windows program. The image area was 768 × 576 pixels and 1 pixel corresponded 1.06 μm. Two images of the transverse section were taken and analyzed in each growth ring formed in 1995, 1996, and 1997. The images were taken at a distance of 200 μm from the outer border of each annual ring. However, when growth ring width was less than 0.6 mm, the image represented the whole growth ring width. At least 100 vessels were measured in each growth ring of the saplings. The vessels that were shown in the image only partly were not included in the vessel lumen diameter measurements, but they were included in the vessel area measurements. The mean of each vessel lumen diameter was measured at 5° intervals around the centroid of the vessel lumen. Altogether 170 samples were analyzed.

Statistical analysis

Data on height, diameter, number of current branches, total biomass, annual increment, vessel lumen diameter, and vessel area were analyzed (SPSS-Win 9.0, general linear models procedure) using a mixed-model four-way analysis of variance (ANOVA) with year, defoliation, and fertilization as fixed effects and clone as a random effect. The annual increment in 1994 was used as covariate in the analysis of annual increment. Distance from the pith was used as covariate in the analysis of vessel lumen diameter and vessel proportional area. The relationship between vessel lumen diameter and distance of the sample from the pith was analyzed with simple linear regression. Data on total leaf area, specific leaf area, leaf size, leaf area ratio, xylem water content, and xylem basic density were analyzed by three-way ANOVA with defoliation and fertilization as fixed effects, and clone as a random effect. Differences among the treatments were analyzed by pairwise comparisons with Bonferroni's test using the estimated marginal means of data to correct any discrepancies resulting from incomplete block design (Searle et al. 1980). Biomass allocation was analyzed with multivariate analysis of variance (MANOVA). For all ANOVAs, error terms were determined according to Zar (1984). The data were checked for normality and homogeneity of variances, and if necessary, the values were transformed to satisfy the assumptions of ANOVA.

Results

Growth and branching

Defoliation reduced growth of silver birch (Fig. 2) at both fertilization levels. There was a significant interaction between year and defoliation (Table 1) reflecting the fact that

defoliation effects diminished during the 2 years of the study. One year after defoliation (1996), 50% defoliation decreased height (Bonferroni's test, the mean difference significant at the 0.05 level) similarly to 75% defoliation as compared with controls that were not defoliated (Fig. 2A). In terms of diameter growth there was a statistically significant reduction because of the 75% defoliation treatment (Fig. 2C). The 100% defoliation decreased both height and diameter growth most severely (Figs. 2A and 2C). With respect to height and diameter growth, partial defoliation did not have a statistically significant effect 2 years after defoliation, while the complete (100%) defoliation was still associated with significantly reduced height and diameter growth (Figs. 2B and 2D).

Defoliation decreased the number of current branches at both fertilization levels (Fig. 3). There was a significant three-way interaction among year, fertilization, and defoliation with respect to determination of branch number (Table 1). However, when data of each year were tested separately, no significant interactions between fertilization and defoliation were detected (1996: $p = 0.544$, 1997: $p = 0.075$). One year after defoliation the 50 and 75% defoliations decreased the number of current branches less than the 100% defoliation, which resulted in the greatest reductions (Fig. 3A). Two years after defoliation, only complete defoliation reduced the number of current branches (Fig. 3B).

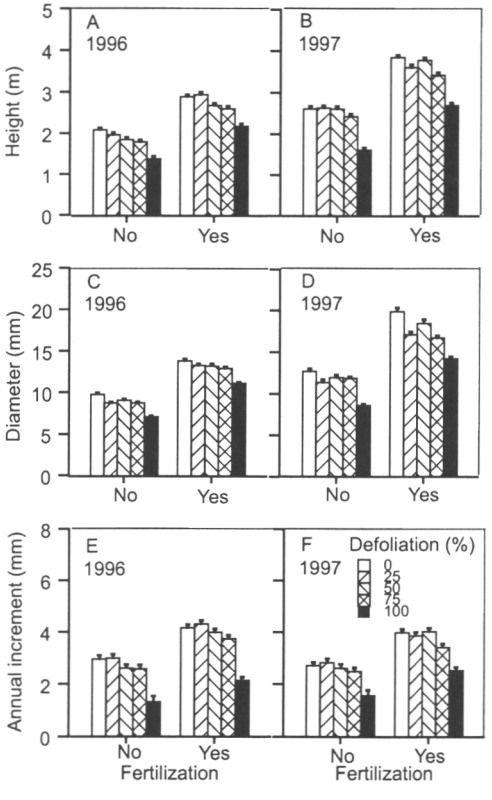
Total aboveground biomass and biomass allocation

Fertilization increased the biomass of saplings approximately threefold as compared with nonfertilized saplings (Table 1, Figs. 3C and 3D). There were significant interactions between fertilization and clone, and among year, fertilization, and defoliation for total biomass (Table 1). When years were analyzed separately, a significant interaction was found for defoliation and fertilization ($p < 0.001$; Fig. 3C).

One year after defoliation, in the nonfertilized saplings, 25% defoliation decreased biomass by the same magnitude as the 75% defoliation, but no significant effect of the 50% defoliation was found (Fig. 3C). The fertilized saplings tolerated the 25% defoliation without significant reductions in biomass, while the 50 and 75% defoliations produced significant decreases in biomass in 1996 (Fig. 3C). The 100% defoliation produced the strongest responses: biomasses were less than half of those of the controls (0% defoliation) at both nutrient levels (Fig. 3C). Two years after defoliation, irrespective of fertilization, some recovery had occurred, but still, the 75 and 100% defoliations clearly continued to reduce the biomass of the saplings (Fig. 3D).

Only the complete (100%) defoliation affected biomass allocation to stem, branches, and leaves (Table 2, Fig. 4). There were significant interactions between year and defoliation for biomass allocation to stem, leaves, and branches (Table 2). One year after the defoliation, biomass allocation to stem increased ($p < 0.01$), but allocation to leaves decreased ($p < 0.001$) in 100% defoliated saplings as compared with controls (Figs. 4A and 4B). After 2 years, however, the opposite pattern was observed. Biomass allocation to stem decreased ($p < 0.001$), and allocation to leaves increased ($p < 0.001$) for the 100% defoliation (Figs. 4C and 4D). Two years after defoliation, there was an interaction between fertilization and defoliation for biomass allocation to branches

Fig. 2. (A and B) Height (m), (C and D) diameter (mm), and (E and F) annual increment (mm) (±SE) of nonfertilized and fertilized *Betula pendula* saplings 1 year (1996) and 2 years (1997) after defoliation. All values are estimated marginal means according to the ANOVA model. For statistics, see text and Table 1.



($p < 0.05$). Biomass allocation to branches tended to increase only in fertilized saplings that were completely defoliated ($p < 0.075$; Fig. 4D).

There were significant interactions between fertilization and clone and between year and fertilization for biomass allocation to stem and branches (Table 2). Biomass allocation to stem increased because of fertilization in the last year of the study ($p < 0.001$) but not in the first year. Increased allocation to branches was detected in fertilized saplings in both years as compared with the nonfertilized saplings ($p < 0.001$ for both years).

Foliage and leaf properties

Complete (100%) defoliation affected leaf properties 2 years after defoliation. Irrespective of fertilization, total leaf area was less than half that of the controls (Table 3, Fig. 5A). Complete defoliation decreased leaf size significantly (Table 3, Fig. 5B). Although there was a significant defoliation effect on the specific leaf area (SLA) (Table 3), no significant differences were detected in pairwise comparisons between the controls and the defoliation treatments; in-

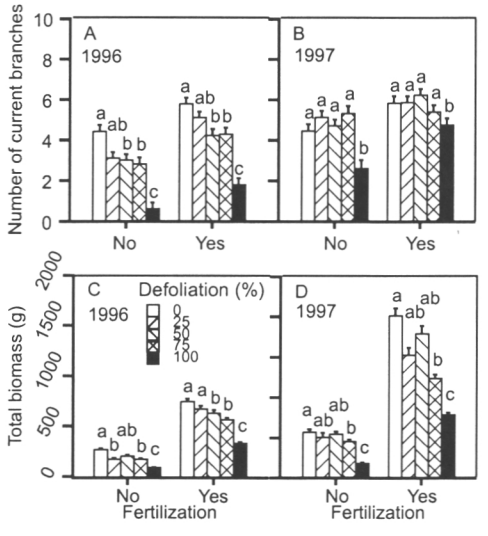
Table 1. ANOVA results for height, diameter, annual increments, current branch number, and biomass of *Betula pendula* in 1996 and 1997.

		F values				
Source of variation	df	Height	Diameter	Increments	Branch no.	Biomass
Year (Y)	1	204.41***	679.55***	0.83	43.80***	511.58***
Fertilization (F)	1	723.49***	644.71***	107.53***	53.31***	766.71***
Defoliation (D)	4	105.19***	56.15***	60.15***	35.20***	113.02***
Clone (C)	14	8.64***	6.98**	2.94*	3.05	3.17*
Block	9	35.26***	27.51***	58.91***	28.11***	41.77***
Covariate (incr. 1994)	1	—	—	10.74**	—	—
Y × D	4	26.12***	2.73*	6.24***	9.42***	3.96**
Y × F	1	43.18***	7.96*	0.41	0.75	0.91
Y × C	14	73.62	—[a]	2.43*	1.65	—[a]
F × D	4	0.70	1.57	0.71	1.01	3.07*
F × C	14	4.15	3.30	1.87	0.89	4.17*
D × C	56	2.12	0.82	0.80	0.67	1.04
Y × F × C	14	0.56	0.40	0.64	1.88*	0.39
Y × D × C	56	0.49	0.59	1.56	1.01	0.56
Y × F × D	4	1.95	0.92	0.80	3.19*	2.63*
F × D × C	56	0.94	1.10	3.11***	1.21	1.27
Y × F × D × C	56	0.94	1.12	0.42	0.96	0.95
Error df		852	852	720	816	845

Note: Data for diameter and annual increments were log transformed. Error terms were year × clone for year, fertilization × clone for fertilization, defoliation × clone for defoliation, year × defoliation × clone for year × defoliation, year × fertilization × clone for year × fertilization, fertilization × defoliation × clone for fertilization × defoliation, year × fertilization × defoliation × clone for year × fertilization × defoliation. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

[a]Values were not calculated by SPSS for Windows version 9.0 programme.

Fig. 3. (A and B) Number of current branches and (C and D) total biomass (g) of nonfertilized and fertilized *B. pendula* saplings 1 year (1996) and 2 years (1997) after defoliation. All values are estimated marginal means. Error bars are SEs. Values with different letters are significantly different ($p < 0.05$) among the treatments.



stead, there was a significant difference between the 50 and the 100% defoliations (Fig. 5C). We also detected a significant interaction between fertilization and defoliation for LAR (Table 3). Altough 100% defoliation increased LAR at both fertilization levels, in the nonfertilized saplings, complete defoliation differed from all other treatments, while in the fertilized saplings, complete defoliation differed significantly only from the controls (Fig. 5D).

Fertilization induced almost a threefold increase in total leaf area by increasing the mean leaf size and by decreasing the SLA and the LAR (Figs. 5A–5D).

Wood properties

Annual increment

The 75 and 100% defoliations decreased the growth ring width of the saplings (Table 1, Figs. 2E and 2F). The differences in annual increments were small among the control, 25, 50, and 75% defoliation treatments (Figs. 2E and 2F). However, there was a significant interaction between defoliation and year (Table 1, Figs. 2E and 2F). The 75 and 100% defoliations reduced the annual increment of the saplings significantly compared with the control during the first year after defoliation (in 1996). Two years after defoliation (1997), only the 100% defoliation caused reduction in the annual increment (Table 1, Figs. 2E and 2F). Fertilization increased annual increment at all defoliation levels (Table 1, Figs. 2E and 2F). The proportional response of the 100% defoliated saplings to fertilization was slightly stronger than in other defoliation levels (Table 1, Figs. 2E and 2F).

Moisture content and basic density

Defoliation had no significant effect on the moisture content of secondary xylem of the saplings, whereas fertilization increased the moisture content significantly from 68.9 ±

Table 2. MANOVA results for the effects of year, fertilization, defoliation, and clone on the allocation of biomass to stem, leaves, and branches in *Betula pendula*.

		F values			
Source of variation	df	MANOVA (Wilks' lambda)	Stem	Leaf	Branch
Year (Y)	1	145.20***	25.81***	362.80***	99.56***
Fertilization (F)	1	123.93***	11.92**	285.24***	106.00***
Defoliation (D)	4	2.18*	1.42	3.79**	1.56
Clone (C)	14	18.47***	34.37***	34.23***	21.00***
Block	9	9.70***	8.95***	17.34***	10.48***
Y × D	4	13.52***	12.08***	39.73***	2.97*
Y × F	1	6.78***	8.53**	1.51	9.30**
Y × C	14	2.97***	2.19**	5.23***	1.68
F × D	4	1.89*	0.69	0.60	2.93*
F × C	14	2.21***	2.09*	0.84	2.88***
D × C	56	0.97	0.97	0.81	0.86
Y × F × C	14	1.25	1.18	1.22	1.16
Y × D × C	56	0.87	0.83	0.53	0.72
Y × F × D	4	1.46	0.60	2.38*	0.71
F × D ×	56	0.94	0.66	0.86	0.67
Y × F × D × C	56	0.74	0.70	0.51	0.67
Error	843				

Note: Data were transformed using arcsine of square roots of proportional values. See Table 1 for error terms. Error df for univariate analysis = 845. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

0.7% (mean ± SE) of dry mass in nonfertilized to 71.1 ± 0.6% in fertilized saplings (Table 4; data not shown).

The differences in basic density of wood were small among the defoliation treatments, but a significant increase in the basic density was found resulting from the 75% defoliation as compared with the controls (Table 4). The wood of nonfertilized saplings was denser than the wood of the fertilized saplings (Table 4). Estimated marginal mean values for basic density of nonfertilized saplings were 424.8 ± 2.3, 432.4 ± 2.3, 431.1 ± 2.2, 435.7 ± 2.4, and 432.8 ± 3.2 $kg \cdot m^{-3}$ for 0, 25, 50, 75, and 100% defoliation, respectively. Estimated marginal mean values for basic density of fertilized saplings were: 414.3 ± 2.2, 415.4 ± 2.2, 417.2 ± 2.1, 420.5 ± 2.2, and 413.4 ± 2.1 $kg \cdot m^{-3}$ for 0, 25, 50, 75, and 100% defoliation, respectively.

Wood structure: vessel lumen diameter and vessel area

There were significant interactions between year and fertilization and between fertilization and defoliation for vessel lumen diameter (Table 5). In nonfertilized saplings the 100% defoliation decreased vessel lumen diameter in relation to distance from the pith (Table 5, Fig. 6A), which was not found in fertilized saplings (Fig. 6B). No simple linear relation between vessel lumen diameter and distance from the pith was detected for the defoliation treatment. The mean vessel lumen diameter was slightly larger in the fertilized saplings than in the nonfertilized saplings and there was a positive linear relation between vessel lumen diameter and distance from the pith in the control saplings (Table 5, Figs. 6A and 6B).

A significant interaction between defoliation and year was detected in vessel proportional area (Table 5). In the annual ring of 1995, the vessel area of completely defoliated saplings was larger than that of the controls (Table 5, Figs. 6C and 6D). One and 2 years after leaf removal (growth rings of 1996 and 1997), the control saplings had slightly larger vessel proportional area than the defoliated saplings (Table 5, Figs. 6C and 6D).

In the 1995 latewood, a zone of wood with defects was seen in the transverse sections of the completely defoliated

Fig. 4. Biomass allocation to stem, leaves, and branches in the different defoliation treatments of nonfertilized and fertilized *B. pendula* saplings (A and B) 1 year (1996) and (C and D) 2 years (1997) after defoliation. For statistics, see text and Table 2.

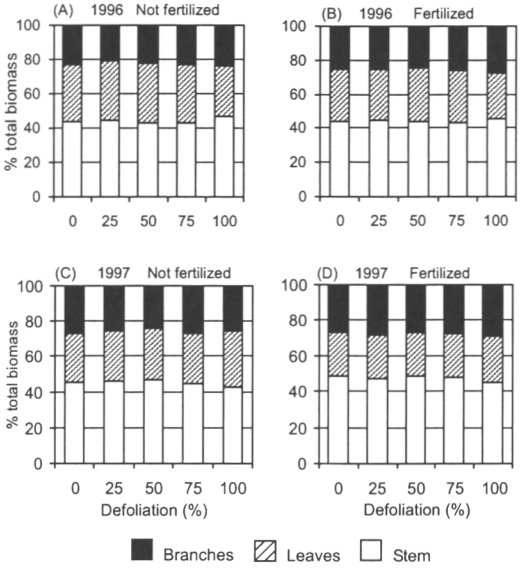


Table 3. ANOVA results for leaf area, leaf size, specific leaf area (SLA), and leaf area ratio (LAR) of *Betula pendula* in 1997.

		F values			
Source of variation	df	Leaf area	Leaf size	SLA	LAR
Fertilization (F)	1	378.85***	51.40***	52.53***	169.83***
Defoliation (D)	4	38.01***	10.49***	2.88*	11.47***
Clone (C)	14	4.16	16.44***	33.71**	18.23**
Block	9	24.28***	29.19***	10.48***	16.71***
F × D	4	1.64	0.74	0.80	2.82*
F × C	14	1.28	1.24	0.61	1.13
D × C	56	0.50	0.84	0.82	0.73
F × D × C	56	1.19	0.78	0.99	0.80
Error df		408	408	408	405

Note: Data for leaf area were log transformed. Error terms are fertilization × clone for fertilization, defoliation × clone for defoliation, and fertilization × defoliation × clone for fertilization × defoliation. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

saplings. This zone was approximately 100 μm wide and consisted of "normal-shaped" thin-walled and sometimes incompletely lignified cells. The wood with defects could be detected as a light blue zone in the safranin – alcian blue stain as well as under fluorescence microscope (Olympus BX60, Olympus URFLT lamp with U-MWU2 excitation cube) as a thin-walled zone showing incomplete lignification.

Discussion

Fertilization increased growth of silver birch saplings at all defoliation levels, demonstrating that nutrient availability limited growth in the experimental field. Irrespective of fertilization, saplings were able to tolerate loss of every fourth leaf (25% defoliation) without an effect on height growth. Reduction of height growth in response to 50 and 75% defoliation was of a similar magnitude (approximately 10%) as was observed in 3-year-old partially defoliated silver birch (Augner et al. 1997). After repeated complete defoliations and topping of juvenile *Betula pubescens* Ehrh., height reduction was stronger (Hjälten et al. 1993) than in the present study. Younger saplings of the same clones showed slighty higher reductions (approximately 16%) in their relative height increase as a response to defoliation conducted earlier in the growing season (Mutikainen et al. 2000). In the present study, older saplings were likely to have increased their storage capacity (Höll 1997), thus enabling better recovery from defoliation.

In terms of diameter growth, saplings tolerated higher defoliation level (50% defoliation, i.e., removal of every other leaf) than in terms of height growth. During a growing season, height growth ceases earlier than diameter growth (Luoranen 2000), and thus, the differing effects of previous-year defoliation on height and diameter growth might be associated with the different growth rhythm. Earlier growth cessation shortens the actual recovery time for utilization of reserves and current resources. In 30-year-old mountain birch (*Betula pubescens* ssp. *tortuosa*), 50% defoliation had no significant effect on annual increment, but in 100% defoliated trees the radial growth during the following 3 years was more severely reduced (Hoogesteger and Karlsson 1992) than in silver birch of our study.

In our study the leaves that were mainly source leaves were removed in the middle of the growing season. This is likely to have disturbed the developmental and maturation processes of the resource sinks (axillary buds). This probably impairs the capability to produce new branches in the following growing season. Our results show that the saplings tolerated removal of only 25% of their leaves in previous year without reduction of current branch number. Two years after the leaf removal the partially defoliated saplings had reached the branch number of controls suggesting an activation of previously supressed meristems, which is a prerequisite for recovery capacity from defoliation (Thomas and Watson 1988), while the completely defoliated saplings had not. In mountain birch that is adapted to poorer soils, recovery was not so obvious as in silver birch in the present study, since reduction of long shoot (branches) growth resulting from cumulative 75% defoliations lasted for more than 2 years (Kaitaniemi et al. 1999).

Marked reduction of total biomass resulting from defoliation was evident. This has also been reported in juvenile silver birch plants 1 and 2 years after damage (Hjälten et al. 1993) and in subarctic dwarf birch (*Betula nana*) and mountain birch 1 year after defoliation (Eckstein et al. 1998). In the present study a slight but significant indication of interaction between defoliation and nutrient availability on total biomass was found 1 year after defoliation. Total biomass of nonfertilized saplings was reduced even by the mildest (25%) defoliation, while fertilized plants were able to withstand a similar leaf loss without impairments. This is in accordance with the suggestion that the probability of compensation in terms of biomasses decreases when nutrient availability decreases (Maschinski and Whitman 1989). The present result differs from those of our earlier study with younger saplings of the same clones (Mutikainen et al. 2000). In the present study the saplings were older, the period between planting and defoliation was longer, the saplings had been fertilized for longer time, and time for recovery was longer, which might explain the discrepancies between the two studies. In deciduous trees, resources are remobilized from senescing leaves to the branches, stem,

Fig. 5. (A) Leaf area (m^2), (B) leaf size (cm^2), (C) specific leaf area (SLA, $cm^2 \cdot g^{-1}$), and (D) leaf area ratio (LAR, $cm^2 \cdot g^{-1}$) of nonfertilized and fertilized *B. pendula* saplings 2 years (1997) after defoliation. Error bars are SEs. All values are estimated marginal means. (A–C) For statistics, see text and Table 3. (D) Values with different letters are significantly different ($p < 0.05$) among the treatments.

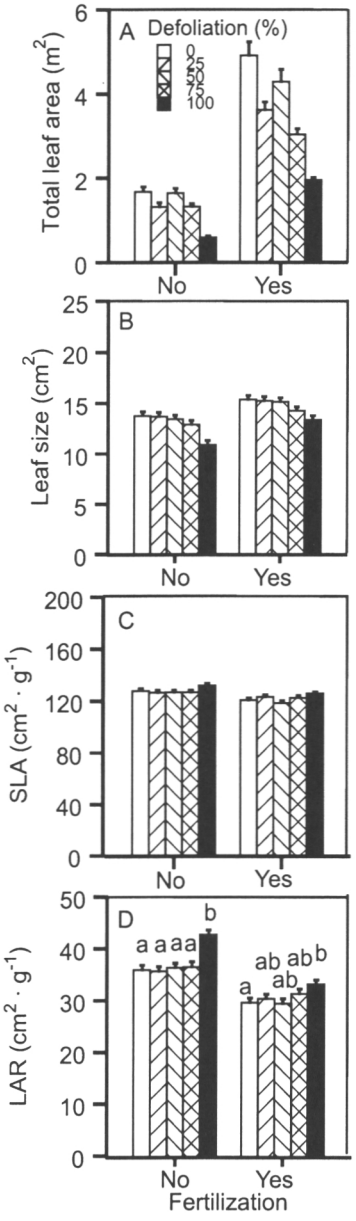


Table 4. ANOVA results for moisture content and basic density of *Betula pendula* stemwood in 1997.

		F values	
Source of variation	df	Moisture	Density
Fertilization (F)	1	9.46**	90.69***
Defoliation (D)	4	0.79	3.02*
Clone (C)	14	3.42**	25.70***
Block	9	22.19***	36.35***
F × D	4	1.14	1.16
F × C	14	0.75	1.73
D × C	56	1.72*	1.08
F × D × C	55	0.77	0.79
Error	366		

Note: See Table 3 for error terms. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

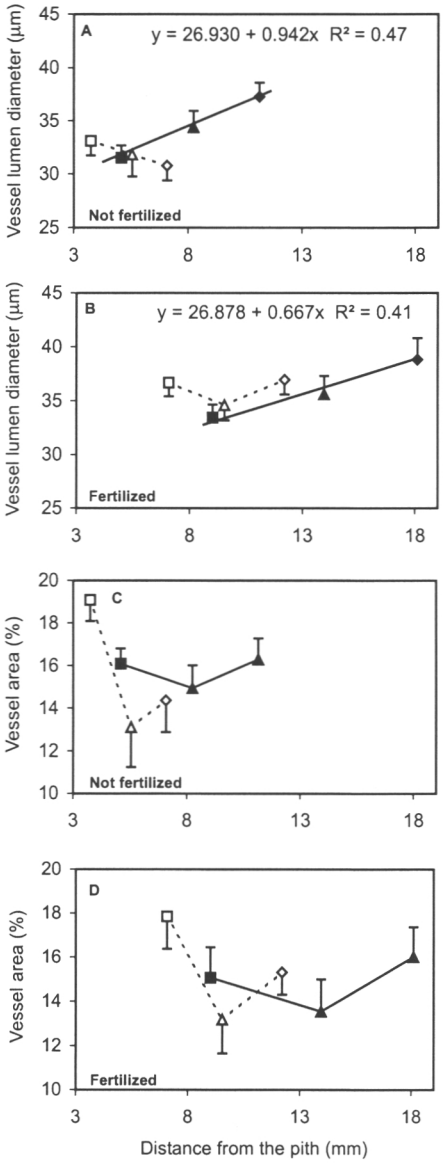
Table 5. ANOVA results for vessel lumen diameter (VLD) and vessel proportional area (VPA) of *Betula pendula* stemwood.

		F values	
Source of variation	df	VLD	VPA
Year (Y)	2	4.40*	29.16***
Fertilization (F)	1	3.61	3.86
Defoliation (D)	1	6.85*	7.80**
Clone (C)	3	3.80	13.02*
Block	6	2.63*	2.62*
Covariate (distance)	1	41.49***	3.16
Y × D	2	4.90*	50.45***
Y × F	2	2.55	2.62
Y × C	6	5.20	—
F × D	1	137.13***	2.02
F × C	3	—	1.69
D × C	3	—	0.75
Y × F × C	6	0.62	0.66
Y × D × C	6	0.79	0.13
Y × F × D	2	2.00	0.94
F × D × C	3	0.03	3.28
Y × F × D × C	6	0.95	0.39
Error	107		

Note: Only 0% and 100% defoliation levels. See Table 1 for error terms and missing values. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

and roots in the autumn (Kozlowski 1992). The remobilized resources are used for regrowth in the following spring (Millard et al. 1998). In the present study, the stronger responses to defoliation in nonfertilized saplings might be explained by impairment of resource remobilization. However, our results showed that the dependence of defoliation effects on nutrient availability disappeared 2 years after the defoliation. This is an interesting finding, and further studies would be necessary to explain why nutrient availability had only a minor role in compensatory growth after defoliation and if there is interaction between nutrients and for example timing of defoliation or physiological status of the plant. However, our results show that in terms of total biomass, 6-year-old silver birch saplings were not able to recover from a single,

Fig. 6. (A and B) Vessel lumen diameter and (C and D) vessel proportional area in nonfertilized and fertilized *B. pendula* saplings 2 years (1997) after 0% (solid line) or 100% defoliation (broken line). The mean vessel lumen diameter (μm; values based on raw data) and vessel proportional area (% of total transverse area; values based on raw data) in annual ring 1995 (squares), 1996 (triangles), and 1997 (diamonds) are presented against the mean distance of the annual ring from the pith ($n \geq 9$). Error bars are SEs. For linear regression equations of nonfertilized control saplings $F = 38.43$ and $P < 0.0001$ and of fertilized control saplings $F = 32.42$ and $P < 0.0001$. For further statistics, see Table 5.



severe (75–100%) defoliation in 2 years irrespective of fertilization level.

Defoliation is likely to affect the regulation of resource flow within a sapling by altering the absolute or relative strength of sinks and sources (e.g., Honkanen et al. 1994). In the present study, removal of all leaves increased allocation to stem at the expense of leaves in the next growing season, i.e., stems maintained their absolute biomass, but proportional stem biomass increased since leaves did not maintain their absolute biomass. Accordingly, in mountain birch, Ruohomäki et al. (1997) reported that in post-treatment year, absolute leaf biomass decreased in shoots that developed from axillary buds whose supporting leaves had been defoliated in previous year. In birch, growth of new leaves in the spring relies on buds that have been developed during the previous summer, since long shoot leaves support their axillary buds (Haukioja et al. 1990). The prerequisite for recovery capacity of plants from defoliation is the activation of previously suppressed meristems, which increases the number of resource-collecting organs (Thomas and Watson 1988). Such a phenomenon was found in our study 2 years after the defoliation, when allocation to leaves increased while allocation to stem decreased. Our results are in accordance with the idea that biomass allocation reflects dynamic change and different timing to restore different allometric relationships after defoliation (Oesterheld and McNaughton 1991). Defoliation increased biomass allocation to branches only in fertilized saplings, which has also been reported earlier (Ovaska et al. 1993), suggesting that in resource-rich conditions there is enough carbon and nutrients to be allocated to branch growth.

In the present study, 2 years after the defoliation, decrease in leaf area and increase in LAR were evident. This has also been reported to occur in the subarctic mountain birch and *B. nana* 1 year after defoliation (Ruohomäki et al. 1996; Eckstein et al. 1998) or after cumulative defoliations (Kaitaniemi et al. 1999). Increase in LAR suggests that in the defoliated saplings, less aboveground biomass per leaf area was supported, i.e., the production of photosynthetic tissue had priority over the other parts of a sapling. This is in accordance with the observed increase in biomass allocation to leaves 2 years after the defoliation. The increase in LAR was slightly lower in the fertilized saplings than in the nonfertilized saplings after defoliation possibly because increased biomass allocation to leaves was accompanied with increased allocation also to branches in fertilized saplings 2 years after defoliation.

In our study, the lack of interaction between defoliation and clone suggests that genetic variation did not play an important role in the tolerance to defoliation (Strauss and Agrawal 1999). However, interactions between fertilization and clone for the total biomass and the allocation of biomass to stem and branches suggest that there was genetic variation in the ability of these birch clones to utilize nutrients. Genetic variation in the ability to use nutrients may result in genetic differentiation between nutrient regimes, as has been suggested in different willow genotypes (Hakulinen et al. 1995).

In our study, a single defoliation event did not have any remarkable effects on basic physical properties of wood of silver birch saplings, except on wood density. The latter was

slightly higher in 75% defoliated saplings than in control saplings. Complete defoliation did not show significant increase in density, because the diameter growth and growth ring width were severely reduced. However, pruning or removing living branches is known to improve the strength properties and quality of stemwood by narrowing annual rings and increasing tracheid length and wood density (Singh and Desai 1998). For conifers, a negative relation between growth ring width and basic density has been detected. For example, fertilization and thinning increase growth ring width, but decrease wood density in Norway spruce (*Picea abies* (L.) Karst.) (Olesen 1976; Saranpää 1994; Dutilleul et al. 1998; Saranpää et al. 2000). This was also the case in the present study, since fertilized saplings with wider growth rings had lower basic density than nonfertilized saplings.

The basic density of silver birch stemwood increases with increasing age and distance from the pith (Hakkila 1966; Bhat 1980). In silver birch the basic density varies between 430 and 570 $kg \cdot m^{-3}$ depending on tree age (Bhat 1980), but in juvenile wood, values less than 400 $kg \cdot m^{-3}$ have been measured (Helińska-Raczkowska 1996). In the present study, the saplings were 6 years old in 1997, and thus, the mean basic density measured (420 $kg \cdot m^{-3}$) was typical of juvenile wood.

Complete defoliation seemed to decrease vessel diameter in silver birch saplings (Figs. 6A and 6B, Table 5). In the juvenile wood of birch the vessel lumen diameter is known to increase with increasing distance from the pith (Fabisiak and Helińska-Raczkowska 1995, 1997). In our study, growth ring width and the vessel lumen diameter decreased in nonfertilized saplings that were completely defoliated. Because of retarded growth of the defoliated saplings the vessels were located closer to the pith than in control saplings (Figs. 6A and 6B). In young trees, slow growth rate leads to delayed maturation. Therefore, the vessel lumen diameter was smaller and vessel proportional area larger in the completely defoliated saplings than in the control saplings.

The interaction between defoliation and fertilization for the vessel lumen diameter can be explained by the fact that vessel lumen diameter tended to decrease in nonfertilized saplings. In contrast, a slight increase in vessel lumen diameter in annual ring 1997 was observed in fertilized saplings, which may be a sign of recovery but may also be caused by the faster maturation of the fertilized saplings. The largest vessel area was measured in the completely defoliated saplings in annual ring 1995. These vessels were located closest to the pith because of the narrow annual ring. This is in accordance with previous studies in silver birch that show a decrease in stemwood vessel proportion, when age and distance from the pith increases (Bhat and Kärkkäinen 1981).

In conclusion, we found no general threshold defoliation level for negative effects of defoliation to occur, since the sensitivity of saplings to defoliation varied according to different plant traits studied. However, the responses were related to the defoliation intensity.

Our results suggest that nutrient availability played only a minor role, whereas the defoliation level and recovery time played more critical roles in compensatory growth of birch saplings. Taken together, these results emphasize the importance of measuring several traits and using a time scale longer than one season when examining the responses of perennial plants to defoliation. Further, they also indicate that we need to consider genetic, environmental, and physiological factors simultaneously.

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ORIGINAL ARTICLE

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Variation of non-structural carbohydrates in silver birch (*Betula pendula* Roth) wood

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Abstract Non-structural carbohydrates in silver birch (*Betula pendula* Roth) wood were analysed in a 7-year-old clone and in five mature stems. The analysis was conducted to obtain more detailed information on seasonal fluctuation of these components and of the tree-to-tree variation and within stem variation. The sugars were analysed by GLC-MS. The smallest total soluble sugar amounts (consisting of sucrose, fructose, glucose, raffinose and *myo*-inositol) in young trees were measured during mid-summer (ca. 0.3%) and the largest while in dormancy (ca. 1.6% on wood dry weight basis). Raffinose was detected in autumn as a minor component. The proportion of monosaccharides and the amount of *myo*-inositol were largest during growth. Compared to other studies silver birch showed more evident seasonal fluctuation in soluble sugars than evergreen tree species. The sugar amount in mature stems was approximately at the same level as in young trees that had the same felling time. Tree-to-tree variation in the non-structural carbohydrates in the mature wood was fairly large. However, the amount of total soluble sugars, sucrose and glucose showed significant variation within the stem. The amount of these sugars was largest in samples that were taken close to the cambium. Starch was also detected close to pith. According to the heartwood definition and starch measurement results in this paper, it could be stated that silver birch does not form heartwood.

Keywords *Betula pendula* · Soluble sugars · Starch · Secondary xylem

Introduction

The most obvious use of carbohydrate reserves in the secondary xylem of trees is in the maintenance of respiration and growth at times when they are not driven by photosynthesis (Hansen and Grauslund 1973; Glerum and Balatinecz 1979; Kramer and Kozlowski 1979; Höll 1997). However, the increase of soluble sugars in the secondary xylem during late fall has also been connected with frost hardening (Höll 1981; Bonicel et al. 1987; Sauter 1988), although the entire mechanism causing increases in soluble sugars during early winter (primarily sucrose and its galactosides) is not fully understood (Sauter et al. 1996). Lastly, the storage and mobilisation of non-structural carbohydrates may also have an immediate effect on biomass development in trees (Chapin et al. 1990; Abod et al. 1991).

Seasonal cycles in the accumulation of storage components tend to be more pronounced in deciduous than in evergreen species (Kramer and Kozlowski 1979). Due to the lack of 2nd and 3rd year needles, which can function as storage tissues in softwoods, the storage components in the secondary xylem are more important for deciduous than for evergreen tree species.

Reserve materials are mainly stored in living cells. In the wood of silver birch soluble sugars and starch are mostly located in axial and ray parenchyma cells. Seasonal fluctuation and partitioning of storage carbohydrates in the secondary xylem has been particularly well defined in many deciduous trees of the temperate zone. However, most studies in the fluctuation of low-molecular-weight carbohydrates have concentrated on branches (e.g. in *Betula populifera* Marsh., Gibbs 1940; and in *Betula pendula* Roth, Sauter and Ambrosius 1986) or on young 1- to 4-year-old stems (e.g. in *Populus* × *canadensis* Moench '*robusta*', Sauter and van Cleve 1994; and in *Tilia* and *Betula*, Abod and Webster 1991) whilst only a few studies have concentrated on mature stemwood (Lindberg and Selleby 1958; Höll 1981).

The purpose of this investigation was to study the seasonal variation and longitudinal and radial distribution of non-structural carbohydrates in silver birch wood in order to obtain basic information about sugar fluctuations that could be utilised in the processing of wood. For example, by selecting correct felling time it might be possible to avoid high sugar concentrations that could

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cause discoloration in wood during timber drying. The present study concentrates on the seasonal variation in storage carbohydrates (e.g. soluble sugars and starch) in the secondary xylem of young silver birch stems. The reason for using a 7-year-old clone instead of mature stems was to avoid taking either increment borings or branches repeatedly from the same stems. Instead, the young trees were sampled throughout one growing season, while the partitioning of storage carbohydrates in the secondary xylem was studied in mature silver birch stems. The role of these reserve components in wood ageing (and in possible heartwood formation) is discussed.

Materials and methods

Plant material

The material for this study was collected during 1996 and 1997 from silver birch (*Betula pendula* Roth) stems and young trees growing in Punkaharju, Laukansaari, Finland. Two stems were felled from a 35-year-old stand and three from a 70-year-old silver birch stand (61°47′N, 29°17′E). The young trees were felled from a 7-year-old silver birch clone (61°48′N, 29°20′E). The number of degree days (d.d.) in 1997 in Punkaharju was 1,326, which was slightly more than the average of the previous 30 years (1,241).

To study the seasonal variation in the amount of non-structural carbohydrates, a silver birch clone (V5952, micropropagated by Stora Enso Ltd in 1990) was used (Table 1). The studied clone was growing in fertile, formerly agricultural and land where the spacing of the trees was 1×1 m. Five young trees were sampled six times: (1) while dormant (7 March 1997), (2) before bud burst (6 May 1997), (3) 2 weeks after bud burst (21 May 1997), (4) during growth (4 July 1997), (5) at the beginning of leaf senescence (15 September 1997) and (6) at the beginning of dormancy (4 November 1997). On each occasion, five saplings were chosen randomly, felled and a 50-cm-long section of the stem was sawn at the height of 1 m, which was above the crown limit. The samples were immediately transported to a freezer in containers (+4°C) and stored at –20°C. Two 1-cm-thick sample disks were also sawn at a 10-cm distance from both ends of the section and combined to form a single sample. The disks were debarked and the cambium was removed carefully.

To study the horizontal variation in the amount of non-structural carbohydrates in the stem, two silver birch stems were felled on 28 June 1996 (trees 1 and 2) and three additional stems (trees 3, 4 and 5) on 4 July 1997 (Table 2). Samples from the stems were sawn at heights of 0 m, 1 m, 6 m and 12 m. In addition, a narrow strip was sawn through the pith immediately after the trees were felled. These 5-cm narrow strips were taken in an east to west direction in trees felled in the summer of 1997. The samples were immediately transported to a freezer in containers (+4°C) and stored at –20°C. The samples were recovered from three zones: (1) 1–3 cm from the pith, (2) 3–6 cm from the pith and (3) outermost sapwood; all samples were separated from both sides of the pith. The two samples taken from different sides of the pith were mixed and combined to form a single sample.

The wood samples were cut into pieces, lyophilised (–60°C, 4 days) and homogenised into a fine powder with a Polymix (Kinematica) mill (–30°C). For enzymatic hydrolysis and gas-chromatographic determination of starch, the homogenisation efficiency was tested. The samples were first homogenised with the Polymix (Kinematica) mill (–30°C) for 5 min and then with a mortar and pestle in liquid nitrogen. No significant differences were detected between starch amounts in samples that were ground only in a Polymix (Kinematica) mill or by both methods.

Table 1 The variation of stem height and growth in the 7-year-old silver birch clone. (*DIAM* diameter at 1 m height in the stem, *GRW* growth ring width at 1 m height in the stem, *SE* standard error, based on rings at 1 m height in the stem excluding the forming ring, *CV* coefficient of variation as percentage, based on rings at 1 m height in the stem excluding the forming ring)

	Average	SE	CV
Height (m)	5.39	0.28	5.15
DIAM (cm)	3.30	0.02	0.55
GRW (mm)	3.16	0.08	2.67
n	30	30	30

Table 2 Tree description of the mature stems. (*DBH* diameter at breast height, *GRW* average growth ring width at 1 m height in the stem, *SE* standard error, based on growth rings measured at 1 m height in the stem)

Tree	Height (m)	Age	DBH (cm)	GRW (mm)	SE (mm)	Crown height (m)
1	20.9	27	22.9	4.36	0.26	4.5
2	22.6	34	19.3	2.96	0.24	9.8
3	26.3	73	27.0	1.88	0.09	11.9
4	22.5	69	22.0	1.56	0.09	8.5
5	23.6	69	21.0	1.56	0.05	10.8

Extraction of soluble sugars

Wood powder (100 mg) was extracted with 2 ml of 80% ethanol first in an ultrasonication bath for 45 min and then for 18 h at +23°C (Mason and Slover 1971). Three parallel extractions were made per wood sample. Phenyl-β-D-glucopyranoside was added to the extraction solution as an internal standard (Marcy and Carroll 1982). After the extraction process, the samples were centrifuged in a Jouan B4 centrifuge (3,500 rpm, 6 min). One millilitre of the sample solution was evaporated dry under a nitrogen gas stream at +40°C. Recovery of the soluble sugars in the extraction process was tested in one sample by adding phenyl-β-D-glucopyranoside, D-glucose, fructose, *myo*-inositol, sucrose and raffinose as internal standards. The recovery of all other sugars tested was over 90%, except for raffinose, which had a recovery of 55%.

Extraction and enzymatic hydrolysis of starch

The wood powder was first heated at 100°C for 10 min to halt enzyme activity. Fifty milligrams of wood powder was extracted with 450 µl of 0.05 M citrate buffer, pH 4.6, and 50 µl of amyloglucosidase (ca. 7 units, EC 3.2.1.3, 102 857, Boehringer Mannheim) and incubated at 37°C for 24 h. Three parallel extractions were made for each wood sample. During incubation the samples were stirred occasionally (Saranpää and Höll 1989). After enzymatic hydrolysis of starch, the samples were centrifuged in an Eppendorf Centrifuge 5417 C (14,000 rpm, 10 min). The hydrolysis efficiency of starch was tested by staining the pellets with potassium iodide-iodine solution (2%, w/v, KI and 1%, w/v, I_2 in distilled water, Wargo 1975) and viewing under a light microscope (Olympus BH-2). Some starch grains remained non-hydrolysed, but longer incubation time did not affect the amount of residual starch. To deactivate amyloglucosidase, the clear supernatant solution was kept at 100°C for 10 min and centrifuged as mentioned above. Forty microlitres of sample solution was used for gas chromatographic determination of glucose. As an internal standard 0.5 ml of 1,000 ppm phenyl-β-D-glucoside solution in 80% ethanol was added to the sample (Marcy and Carroll 1982). The samples were evaporated dry under a stream of nitrogen (+40°C).

Derivatisation and gas chromatography of non-structural carbohydrates

Trimethylsilyl derivatives of non-structural carbohydrates were formed. Samples were evaporated dry, and 400 µl of *N*-trimethylsilylimidazole(T-7510, Sigma Chemical)/pyridine (21:100, v/v) was added. After half an hour of incubation at 80°C, the TMS-derivatised non-structural carbohydrates were subjected to GC-MS (Brittain et al. 1971). A Hewlett Packard gas-chromatograph 5890 series II with a mass spectrometer 5988A was used for the quantitative measurement of soluble sugars and starch. The TMS derivatives of carbohydrates were determined on a 25 m HP-5 (5% phenyl methyl siloxane) column (Hewlett Packard) with an internal diameter of 0.2 mm and a film thickness of 0.33 µm.

The column-temperature program started at 110°C. The temperature increased at a rate of 10°C/min reaching a final temperature of 300°C, which was held for 36 min. Helium with an inlet pressure of 100 kPa served as the carrier gas. A split-injection mode was used. The injection volume was 1 µl and the split flow was 15 ml/min (split ratio 1:15), whilst the septum purge was 3 ml/min. Total ion chromatograms (TIC) of TMS derivatives (carbohydrates) were used for analysis with the mass range being 50–600 ion mass units. TMS carbohydrates were identified by co-chromatography of authentic TMS derivatives by GC-MS (Hewlett Packard, HP 6890, with a mass selective detector, temperature program and gas flow adjustments as above).

Phenyl-β-D-glucopyranoside was used as an internal standard (Marcy and Carroll 1982), whilst fructose, D-glucose, *myo*-inositol, sucrose and raffinose were used as external standards in the quantitative analysis of TMS-derivatives of the non-structural carbohydrates. In addition an external standard solution, which contained silylated standard sugars, was injected after every 15 injected samples.

Protein determination

The wood samples of five mature trees taken at the height of 1 m (1–3 cm from the pith, 3–6 cm from the pith and outermost sapwood) were selected for protein content determination. Three parallel extractions were made for each wood sample. One and a half grams of lyophilised wood powder was extracted twice with 10 ml of 0.05 M TRIS-maleate buffer, pH 7.7 containing 5 mM Na_2EDTA (Titriplex III), 1 mM $CaCl_2$, 10 mM $Na_2B_4O_7$, 1 M NaCl, 1.5% Polyclar AT and protease inhibitor (Complete, Roche Diagnostics, No. 1 697498, 1 tablet/500 ml). The samples were occasionally shaken during extraction. After two extractions for 30 min at +4°C the samples were filtered through Miracloth (Calbiochem) and the supernatants were further filtered through Gelman Acrodisc filters (pore size 0.45 µm) for ion chromatography (Fagerstedt et al. 1998). Protein contents of the filtered extracts were determined with Bio-Rad microassay procedure (Bradford 1976).

Statistics

Statistical analyses were made using SPSS for Windows (Version 8.0.1). Gauss distribution of populations was checked using the Kolmogorov-Smirnov test with Lilliefors significance correction. Homogeneity of variances was studied using Levene's test.

The amount of non-structural carbohydrates in the seasonal fluctuation study followed Gauss distribution, but variance was not equal in the case of *myo*-inositol, total soluble sugars, sucrose or starch. Therefore, both non-parametric and parametric tests were used, and their results were compared. Kruskal-Wallis analysis and one-way ANOVA gave similar results for all dependent variables tested (also, when variances were not equal: *myo*-inositol, total soluble sugars, sucrose and starch). The data were finally analysed with a one-way ANOVA followed by the Tukey test.

The data in the study of the partitioning of non-structural carbohydrates within mature stems also followed Gauss distribution and variance as equal except in the cases of sucrose and total soluble sugars. The data were analysed by multivariate analysis of variance with the General Linear Model. This is composed of repeated measures of analysis including Huynh-Feldt's test and the *t*-test. Note that the *t*-test was only used when there were significant differences in the samples taken from different heights. In the analysis model two internal subject factors and their interaction were tested. Internal subject factors were the height of the samples (4 levels: 0 m, 1 m, 6 m and 12 m) and the distance of the sample from the pith (2 levels: 1–3 cm from the pith and 1–3 cm from the cambium). Only two levels of the distance factor could be tested, because the diameter at 6 m and 12 m heights was much less than at 0 m and 1 m heights and only two samples were taken at the heights of 6 m and 12 m.

The protein analysis data followed Gauss distribution and the variance was equal. The data were analysed by one-way ANOVA.

Results

Silver birch wood contained non-structural carbohydrates, which were identified as starch, sucrose, glucose, fructose, *myo*-inositol, raffinose, galactose, maltose and stachyose. Only trace amounts of galactose, maltose and stachyose (under 0.01 µg/mg of wood on dry weight basis) were detected. Total soluble sugar content was given as the sum of the five dominant sugars measured in the wood (sucrose, glucose, fructose, *myo*-inositol and raffinose). Verbascose, which has been detected in *B. verrucosa* by Lindberg and Selleby (1958), could not be detected by GC-MS with the HP-5 column. *Myo*-inositol was included in the quantitative analysis although it is not a sugar according to its chemical structure.

Seasonal variation

The major non-structural carbohydrates in the secondary xylem of the 7-year-old silver birch clone throughout the year were sucrose, starch, fructose and glucose (Fig. 2 A–C). The amount of total non-structural carbohydrates (soluble sugars + starch) was smallest in July (4.3± 0.2 µg in 1 mg of wood on a dry weight basis ±SE) and largest in November (35.4±0.8 µg of sugar in 1 mg of wood on a dry weight basis ±SE; Figs. 1, 2A, Table 3). The total amount of soluble sugars (sucrose, glucose, fructose, raffinose + *myo*-inositol) followed the trend of

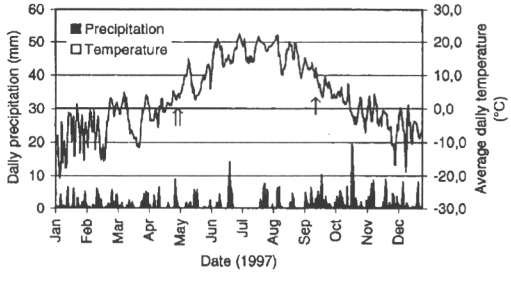

Fig. 1 Average daily air temperatures and daily precipitation during the year 1997 at Punkaharju. The Finnish Meteorological Institute provided data. The arrows indicate the time of bud break (⇑) and leaf yellowing (↑)

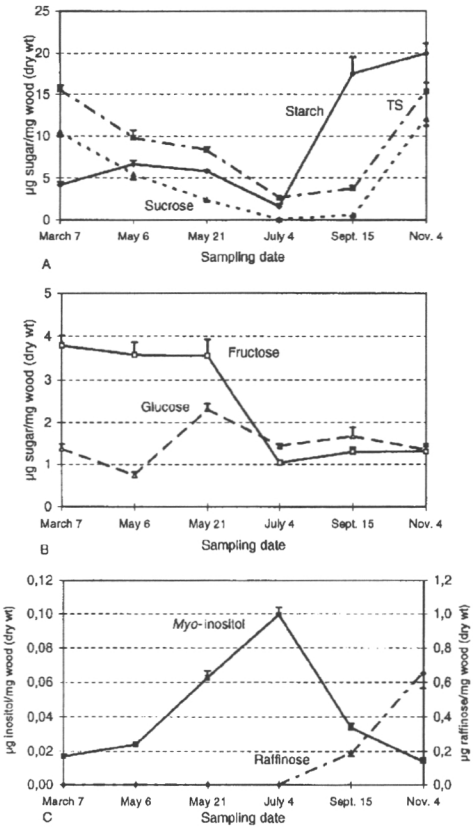

Fig. 2 Seasonal changes in **A** total soluble sugars (– · –), sucrose (···) and starch (—), **B** fructose (—) and D-glucose (– – –) and **C** *myo*-inositol (—) and raffinose (– · –). Each value connected with a line represents the average sugar amount of five independent trees expressed as micrograms of sugar per milligram of wood (on a dry weight basis). Starch is measured as glucose units. Bud break occurred at the beginning of May and yellowing of leaves in the middle of September. *Error bars* indicate tree-to-tree variation (standard error, n=5). For significance of differences, see Table 3

sucrose on all sampling occasions through the year. The amount of sucrose decreased gradually during the spring until July, stayed near the minimum level in September and increased dramatically in November (Figs. 1, 2A, Table 3).

The amount of starch increased slightly during the bud break in May and stayed at the same level for 2 weeks. The amount of starch was smallest (1.6±0.1 μg of glucose in 1 mg of wood on dry weight basis ±SE) in July, and during the autumn it increased gradually until November (Fig. 2A, Table 3).

The amount of monosaccharides was largest 2 weeks after the bud break in May (5.9±0.3 μg in 1 mg of wood on a dry weight basis ±SE) and smallest in mid-summer in July (2.5±0.1 μg in 1 mg of wood on a dry weight basis ±SE). However, the proportion of monosaccharides was about 57% of the total amount of non-structural carbohydrates (soluble sugars + starch) in July (Fig. 2B, Table 3). The amount of fructose in March and May was approximately 3.6±0.2 μg in 1 mg of wood on a dry weight basis ±SE. Before mid-summer the amount of fructose decreased and stayed relatively small in September and November. The amount of glucose decreased slightly before the bud break and increased significantly 2 weeks afterwards. In July the amount of glucose decreased once again (Fig. 2B, Table 3).

In Fig. 2C the seasonal variation in *myo*-inositol and raffinose is presented. In comparison with the amounts of soluble sugars and starch, the amount of *myo*-inositol had a different fluctuation profile. The amount of *myo*-inositol was about three times larger in July during growth than in March, early May, September and November. Raffinose was not detected in March, May or July. The amount of raffinose increased 3-fold from leaf yellowing in September to leaf fall in November. However, raffinose was a minor component of non-structural carbohydrates in November (approximately 2% of total non-structural carbohydrates = soluble sugars + starch; Figs. 1, 2C, Table 3).

Variation within the stem

The sugar composition of mature stemwood was similar to that which was measured on 4 July 1997 in the stemwood from the young trees in the 7-year-old clone. The predominant non-structural carbohydrates were starch, sucrose, glucose and fructose (Fig. 3A–F). The amounts of sucrose, glucose and total soluble sugars (sucrose, glucose, fructose + *myo*-inositol) showed similar distribution patterns and were larger in samples taken from closer to the cambium than in samples taken close to the pith (Fig. 3A–C, Table 4). The average amounts of su-

Table 3 Statistics of seasonal variation data (The same letters on the same row are not significantly different at P<0.001 by Tukey's test)

Sugar						
Starch	a	ab	ab	a	bc	c
Sucrose	a	b	bc	c	c	a
TS	a	b	b	c	c	a
Fructose	a	a	a	b	b	b
Glucose	ab	a	c	ab	bc	ab
Raffinose	a	a	a	a	a	b
Myo-inositol	ab	ab	c	d	b	a
Sampling date	March 7	May 6	May 21	July 4	September 15	November 4

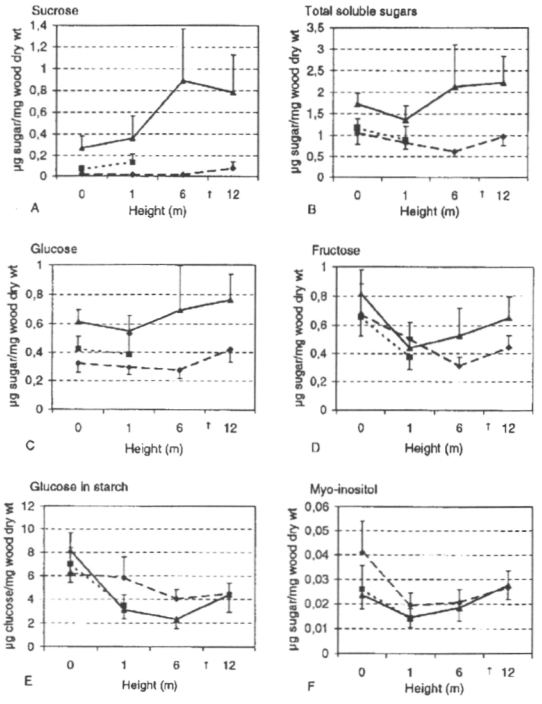

Fig. 3 Partitioning of **A** sucrose, **B** total soluble sugars (containing sucrose, glucose, fructose and *myo*-inositol), **C** glucose, **D** fructose, **E** starch in glucose units and **F** *myo*-inositol within the stem. Each value connected with a line represents the average sugar amount of five independent trees expressed as micrograms of sugar per milligram of wood (dry weight). Each *line* represents distance from the pith: 1–3 cm from the pith (- -), middle part of the wood 6–12 cm from the pith (···) and outermost part of the wood 1–3 cm from the cambium (—). The *arrow* (↑) indicates average crown height of five stems. *Error bars* indicate tree-to-tree variation (standard error, n=5). For statistics, see Results and Table 4

crose at a height of 1 m were 0.02 (±0.001) close to the pith, 0.13 (±0.03) in the middle part of the wood and 0.36 (±0.09) µg of sugar in 1 mg of wood (dry weight) ±SE close to the cambium (Fig. 3A). The amounts of soluble sugars, except fructose, seemed to be larger in the upper parts of the stem than near the stump (Fig. 3A–D, Table 4). However, the amounts of starch and *myo*-inositol seemed to be rather large at stump height (Fig. 3E, F, Table 4).

The amount of starch varied between 18.0 and 0.9 µg in 1 mg of wood dry weight with tree-to-tree variation being large (Fig. 3E, Table 4). In July the average amount of fructose in mature silver birch stems was approximately 0.54±0.04 µg in 1 mg of wood on a dry weight basis (Fig. 3D, Table 4). *Myo*-inositol was the only sugar measured that showed significant variation at different heights of the stem and hence pair-wise comparisons were made (Fig. 3F, Table 4). The amount of *myo*-inositol was larger at the heights of 0 m and 12 m than at the heights of 1 m and 6 m (at P<0.009, when 1 m and 12 m were compared, at P<0.021, when 6 m and 12 m were compared, at P<0.031, when 1 m and 0 m were compared and at P<0.047, when 6 m and 0 m were compared by t-test; for full results, see Fig. 3F, Table 4).

The average protein contents in the five mature trees at 1 m were 0.074 (±0.008), 0.068 (±0.006) and 0.076 (±0.006) µg/mg wood dry weight (±SE, n=5) close to the pith, in the middle and in the outermost sapwood, respectively. The differences in the amounts of proteins in the samples taken at different distances from the pith at a height of 1 m were non-significant.

Discussion

Seasonal variation

Seasonal changes in the amount of total soluble sugars and sucrose in silver birch wood are in agreement with other studies on deciduous trees. In the wood of deciduous trees, stored reserves decrease sharply during the bud break and with the onset of shoot expansion (Kozlowski and Keller 1966). In coniferous trees (e.g. *Pinus sylvestris* L., Fischer and Höll 1992) the changes in soluble sugars were less pronounced than in this study on silver birch. The amount of total soluble sugars in poplar (*Populus* × *canadensis* Moench '*robusta*'), 3-year-old branch wood was at its minimum (2–4 µg of sugar in 1 mg of wood on a dry weight basis) in late

Table 4 Manova results (Huynh-Feldt's test) for sucrose, total soluble sugars (*TS*, glucose, fructose, sucrose and *myo*-inositol), glucose, fructose, starch and *myo*-inositol in mature silver birch trees felled on 4 July. The F values are presented for within subject factors and their interaction with levels of significance indicated

Source of variation	Sucrose			TS			Glucose		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Height	2.29	1.47	0.282	1.60	0.75	0.481	2.11	1.28	0.329
Distance	1.00	5.69	0.076	1.00	7.16	0.055	1.00	7.14	0.056
Height×Distance	2.38	1.24	0.340	2.29	1.12	0.376	1.79	0.48	0.618
	Fructose			Starch			*Myo*-inositol		
Height	1.51	2.74	0.146	1.63	2.27	0.180	1.39	9.62	0.019
Distance	1.00	3.74	0.125	1.00	1.71	0.262	1.00	2.37	0.199
Height×Distance	3.00	2.10	0.154	1.39	4.04	0.089	2.22	2.67	0.121

April/early May during the bud break (Sauter and van Cleve 1994). In the 7-year-old silver birch clone studied, the minimum amount of soluble sugars was detected much later (4 July) than in trees of the temperate zone examined in previous studies (e.g. Sauter and van Cleve 1994). Furthermore, the dramatic increase during autumn in the amount of sucrose was detected in this study earlier than in *B. verrucosa* Ehrh. mature stems (Höll 1981). The reason for these differences is probably the later onset of growth and shorter growth period in the more northern latitudes than in Central Europe. The thermal growing season displays a well-known gradual decrease from south to north (Koski and Sievänen 1985). The annual temperature rhythm is the main regulating factor of the environment (Koski and Sievänen 1985). Thus, seasonal changes in soluble sugars in the silver birch clone studied show a similar overall pattern to other deciduous trees in the temperate zone although the timing is somewhat different depending on the length of the growing season.

The gradual decrease in the amount of total soluble sugars in silver birch wood (on 6 May and 21 May) correlated with the onset of shoot growth (Fig. 2A, Table 3). There was more variation between individual trees in the amount of total soluble sugars (sucrose, glucose, fructose + *myo*-inositol) during the bud break (on 6 May, coefficient of variance, CV=19.7%) than in the samples that were taken 2 weeks later (on 21 May, CV=9.4%). The timing of bud break was probably not synchronised in the 7-year-old silver birch clone although the clone was growing on the same site. Therefore, tree-to-tree variation during this period may exist. Consequently, there can be more variation in the amounts of sugars in the samples that were taken during the bud break.

The slight and non-significant change in the amount of starch after the bud break in silver birch wood is in disagreement with some reports on woody plants. This "springtime starch increase" was not as marked as in other studies (e.g. Sauter and Ambrosius 1986; Fischer and Höll 1992). A prominent resynthesis of starch and a great decrease in sugars in the symplast of silver birch branches has been found to be parallel to the growth and blossoming of catkins (Sauter and Ambrosius 1986). These results were based on a more pronounced change in the amount of measured starch in silver birch branches than in silver birch stemwood during the spring. However, in this study the silver birch clone was only 7 years old and was not yet mature enough to form catkins. In addition, it has been found that in Scots pine sapwood a marked increase in starch occurred at the beginning of the growing season (Fischer and Höll 1992). Unlike deciduous trees, coniferous trees have a functioning photosynthetic system when annual shoot growth begins. Therefore, the "springtime starch increase" can be more pronounced than in deciduous tree species.

The amount of sucrose and raffinose in silver birch wood increased significantly in November, when defoliation had finished. In addition, the largest amount of starch was detected at this time (Fig. 2A, C, Table 3). However, the amount and proportion of raffinose was rather small (5.1% and 4.2% of soluble sugars in September and in early November, respectively). An increase in the amount of soluble sugars, particularly sucrose and its galactosides, has been reported during natural and artificial frost hardening (Nelson and Dickson 1981; Fischer and Höll 1992; Sauter and van Cleve 1994). In late autumn the total amount of soluble sugars in the 3-year-old branch-wood of poplar began to increase, reaching 17–32 µg of sugar in 1 mg of wood on a dry weight basis (Sauter and van Cleve 1994). The increase in sugars has been related to the disappearance of starch (Sauter and van Cleve 1994). The maximum amount of starch has been detected at the time of leaf fall, e.g. in poplar (Sauter and van Cleve 1994). The temperature in Punkaharju, Finland had remained below –2.0°C for the 2 weeks before sampling on 4 November; consequently the silver birches were completely defoliated and dormant (Fig. 1). In Punkaharju, Finland the average duration of the growing season was 162 days (heat sum, >+5°C, d.d. 1,250), which was much less than in Central Europe (202–194 days from latitudes 53° to 47°, heat sum, >+5°C, d.d. varied between 1,650 and 2,030; Koski and Sievänen 1985).

The maximum amount of *myo*-inositol was detected during growth in July (Fig. 2C, Table 3). This non-structural carbohydrate plays a central role in growth and development (Loewus and Loewus 1983) and is involved, for example, in the biosynthesis of cell wall polysaccharides (Roberts and Loewus 1966), in phospholipid metabolism (Dumville and Fry 2000) and in biosynthesis of raffinose series of oligosaccharides (Horbowicz and Obendorf 1994; Loewus and Murthy 2000). The maximal amount of *myo*-inositol in the secondary xylem of young silver birch trees coincided with the rapid growth phase, when the differentiating xylem needs this component. In cyclitol-storing trees like *Acer pseudoplatanus* (quebrachitol), *Quercus robur* (quercitol) and *Fraxinus excelsior* (mannitol) the amount of cyclitol increased in autumn (Popp et al. 1997). In cyclitol storing trees the amount of polyols was considerably larger than the amount of *myo*-inositol in silver birch, where sucrose and its galactosides had taken over the function as osmotica during dormancy.

Variation within the stem

Compared to the amount of non-structural carbohydrates in the young trees on 4 July 1997, the amounts were at the same level in the mature stems, except in the case of *myo*-inositol. In addition, tree-to-tree variation was larger in mature stems than in the 7-year-old clone.

The amounts of sucrose, glucose and total soluble sugars (sucrose, glucose, fructose + *myo*-inositol) in mature stems were largest in samples close to the cambium (Fig. 3A–C, Table 4). Sucrose is the principal form in which fixed carbon and energy are translocated in plants. Therefore, it seems evident that the sucrose gradient in-

creased towards the cambium and phloem in the mature silver birch stems. The mature trees were felled at the moment of rapid growth in mid-summer. Thus, the ray parenchyma close to the cambium was in an active metabolic state. The amounts of sucrose and glucose seemed to be more abundant in the upper parts of the crown, e.g. those parts of the stem that are closer to photosynthesising leaves, where the assimilation of CO_2 takes place.

Heartwood is defined as "the inner layers of wood which, in the growing tree, have ceased to contain living cells and in which the reserve materials (e.g. starch) have been removed or converted into heartwood substances" (Anonymous 1957). In our study starch was detected in all samples of mature silver birch trees (Fig. 3E). According to the seasonal variation study the amount of starch was at its minimum in the mid-summer (Fig. 2A). In spite of its small amount at the time of rapid growth, starch was also detected in samples which were taken close to the pith in the mature wood of silver birch. Furthermore, the amount of starch seemed to be larger close to the pith than close to the cambium at the heights of 1 m and 6 m (Fig. 3E, Table 4).

The differences between the total protein contents in the samples taken at three separate positions form the cambium were very small. Furthermore, we observed 4,6-diamidino-2-phenylindole-stained ray parenchyma cells of a 79-year-old silver birch stem under a fluorescence microscope (Olympus BX-60, excitation cube U-MWU). The observed ray parenchyma cells close to pith contained DNA in compact sickle-like structures. According to our study and based on the definition of heartwood (Anonymous 1957), it can be claimed that silver birch does not form heartwood. Thus, the ray parenchyma cells close to the pith were living, capable of starch biosynthesis and contained approximately the same amount of total proteins as cells close to cambium.

Significant differences in sugar amounts between samples taken at different heights were detected only in the case of *myo*-inositol (Table 4). *Myo*-inositol was present in all the samples of silver birch wood studied (Fig. 3F, Table 4). This cyclitol has been detected in *B. verrucosa* and *B. pubescens* stem-wood (Lindberg and Selleby 1958) and also in the heartwood of some other tree species (e.g. *Sequoia sempervirens*, Anderson et al. 1968; *Planchonella vitiensis*, Cambie et al. 1997). There may be several reasons why the highest amount of *myo*-inositol was detected in samples taken at stump height and in samples close to pith. *Myo*-inositol could in the inner and lower parts of the stem probably serve as a metabolic reserve pool for the more active parts of the stem (wood cells close to cambium).

The absolute values of *myo*-inositol were different when the young and mature trees were compared (Figs. 2C, 3F). This may be related to tree-to-tree variation, which was larger between mature trees than within the clone, or to maturation processes. *Myo*-inositol and starch seem to have almost similar within tree variation profiles, which supports the idea that these non-structural carbohydrates could serve as metabolic reserve components in the mature stems. The smallest amount of *myo*-inositol was detected in the middle of the stem (Fig. 2F). Popp et al. (1997) detected a positive correlation between the amount of quercitol and stem height in *Q. robur*, although they also detected seasonal variation in the quercitol gradients. The biological functions of cyclitols differ from each other. *Myo*-inositol in silver birch could probably serve as a reserve for metabolic intermediates of raffinose family oligosaccharides, which are needed during cold acclimation in autumn.

Conclusions

Large seasonal variation in the amounts of soluble sugar and starch was detected in the secondary xylem of young silver birch trees. Considerable variation in the amounts of non-structural carbohydrates within the tree and between the trees was also detected in 30-year-old stems of the same species. However, no pattern in the radial distribution of non-structural carbohydrates that could be explained by heartwood formation was observed. The enzyme activities of sugar metabolism in ray parenchyma of mature and young silver birch wood and the biochemistry behind the seasonal changes especially in the amounts of sucrose and starch are subjects for further studies.

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IV

Tree Physiology 24, 000–000

Seasonal and within-stem variation of neutral lipids in silver birch (*Betula pendula*) wood

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Summary Neutral lipids were analyzed in stem wood of a 7-year-old clone and in five 35–70-year-old mature trees of silver birch (*Betula pendula* Roth). In young trees and in mature wood of old trees, the free fatty acid fraction comprised less than 5% of the concentration of triacylglycerols (TG). The concentration of free linoleic acid was lowest in March when the young trees were dormant and highest during midsummer and September. In mature trees, the TG concentration increased towards the pith, indicating that living parenchyma cells close to the pith have a large TG storage capacity. The TG concentration (mean 0.51 ± 0.02% of wood dry mass) remained constant throughout the year in young trees, whereas the concentration of β-sitosterol, the dominant free sterol, (mean 82.5 ± 0.4% of total free sterols) decreased during spring and early summer when the temperature gradually increased, and increased during autumn when the trees became dormant. In young trees, we detected a seasonal interconversion between the free and esterified forms of β-sitosterol and campesterol, and within the steryl ester fraction between squalene and betulaprenol-7. The concentration of esterified sterols/isoprenoids was exceptionally high, especially in the inner regions of mature stem wood (mean 0.6 ± 0.03% of wood dry mass). No heartwood formation was detected.

Keywords: deciduous, fatty acid, isoprenoid, sterol, xylem.

Introduction

According to the definition proposed by Sinnot (1918), diffuse-porous angiosperms belong to a tree group whose principal form of stored energy in wood is fat (i.e., triacylglycerols (TG)) instead of starch. However, this definition remains controversial (Ziegler 1964). Studies of diffuse-porous angiosperm tree species have shown that starch is an important storage form of energy in wood of some species (e.g., silver birch (*Betula pendula* Roth), Piispanen and Saranpää 2001) and that conversion of starch to fat is negligible during autumn in the wood of several species (Sauter and van Cleve 1991, Harms and Sauter 1992).

In silver birch, fat stored in wood (mainly TG) is detected as tiny fat droplets in radial and axial parenchyma cells (Harms and Sauter 1992). In herbaceous plant cells, free fatty acids are metabolic intermediates and are not regarded as a storage pool. The size and composition of the free fatty acid fraction in living wood cells depends on the composition of the cell membranes and the TG fraction (Höll and Priebe 1985).

Plant sterols are structural components of plant membranes and play a significant role in the regulation of plant membrane properties (Rodriguez et al. 1985, Rahier and Taton 1997). All plant sterols are able to regulate plasma membrane fluidity, but with different efficiencies, by altering the physical state of the plasma membrane lipid bilayer and restricting the motion of fatty acid chains (Hartmann 1998). Plant sterols have also been shown to modulate ATPase activity in plasma membranes of maize (*Zea mays* L.) roots (Grandmougin-Ferjani et al. 1997).

Sterols serve as intermediates in the synthesis of plant steroid hormones such as brassinolide, which is derived from campesterol (Clouse and Sasse 1998). Recent studies have revealed other functions for plant sterols. For example, β-sitosterol functions as sitosterol-β-glucoside, a glucan polymerization primer in cellulose synthesis (Peng et al. 2002). Steryl esters, which occur in plants in soluble forms, such as in lipoprotein complexes (Wojciechowski 1991, Dyas and Goad 1993, Gondet et al. 1994), are involved in storage and transport. In Scots pine, the concentration of steryl esters increases across the heartwood towards the pith (Höll and Lipp 1987, Saranpää and Nyberg 1987*a*). The accumulation of free sterols in the innermost heartwood has been observed in Scots pine (Höll and Lipp 1987, Saranpää and Nyberg 1987*a*) and black locust (*Robinia pseudoacacia* L., Hillinger et al. 1996).

The lipophilic extractives affect both the chemical and mechanical processing of wood. It is a well known that silver birch (*Betula pendula* Roth), like other diffuse-porous angiosperms (e.g., *Populus tremuloides* Minchx, Chen et al. 1995), causes more pitch problems during Kraft pulping than soft woods like Scots pine (*Pinus sylvestris* L.) (Mustranta et al. 1995). Although the composition of lipophilic extractives in birch wood has been characterized (e.g., Perilä and Toivonen 1958, Selleby 1960, Clermont 1961, Assarsson and Åkerlund 1966, Paasonen 1967, Ekman and Pensar 1973, Höll and Poschenrieder 1975, Harms and Sauter 1992), such studies have been limited to the identification and characterization of

/ Symbols show the height of the lowermost living branch

(crown height) for Trees 1-5.

the extractive components or the samples were taken from branches or from only one tree. Little is known about the seasonal variation in lipophilic components of stem wood.

We studied the seasonal and within-stem variations in the concentrations of the principal lipophilic extractives in silver birch stem wood. We also compared the concentrations of storage lipids, free fatty acids and sterol components in young and mature deciduous hardwood and discussed the physiological roles of lipophilic components in silver birch stem wood. Because repeated increment coring of mature stems would have caused wound reactions and affected the concentrations of the studied components, we used a 7-year-old silver birch clone, thereby minimizing inter-tree genetic variation.

Materials and methods

Plant material

During 1996 and 1997, stem material was collected from 7-, 35- and 70-year-old silver birch (*Betula pendula* Roth) trees growing in Punkaharju, Laukansaari, Finland (61°47′ N, 29°17′ E, mature stems; 61°48′ N, 29°20′ E, young trees). The same samples were also used for a study of soluble carbohydrates (Piispanen and Saranpää 2001). Two trees were felled in a 35-year-old stand and three trees were felled in a 70-year-old stand of silver birch. The young trees were felled from a 7-year-old silver birch clone (V5952, micropropagated by Stora Enso, Stockholm, Sweden, in 1990) stand growing in fertile former agricultural land with a tree spacing of 1 × 1 m. Mean height, diameter at 1 m height and growth ring width at 1 m stem height of the young trees were 5.39 ± 0.28 m (± SE), 3.30 ± 0.02 cm (± SE) and 3.16 ± 0.08 mm (± SE), respectively. The number of degree days in 1997 in Punkaharju was 1326.

To study seasonal variation in lipid concentration, five young trees were felled on each of six dates: (1) while dormant (March 7, 1997); (2) before bud burst (May 6, 1997); (3) two weeks after bud burst (May 21, 1997); (4) during radial growth (July 4, 1997); (5) at the beginning of leaf senescence (September 15, 1997); and (6) at the beginning of dormancy (November 4, 1997). At each sampling, a 50-cm-long section of stem was removed at a stem height of 1 m from each felled tree. The samples were immediately transported to the laboratory in containers (4 °C) and stored at −20 °C. Two 1-cm-thick sample disks were removed at a distance of 10 cm from each end of the section and combined to form a single sample. The disks were debarked and the cambium was removed.

To study within-stem variation in lipid concentration, two 35-year-old silver birch trees were felled on June 28, 1996 (Trees 1 and 2) and three 70-year-old trees (Trees 3, 4 and 5) were felled on July 4, 1997 (for tree characteristics, see Figure 1). Stem samples were removed at heights of 0, 1, 6 and 12 m (Figure 1). In addition, a narrow strip was sawn through the pith immediately after the trees were felled. The samples were immediately transported to the laboratory in containers (4 °C) and stored at −20 °C. Subsamples of these stem samples were obtained from the outermost sapwood and at 1–3 cm

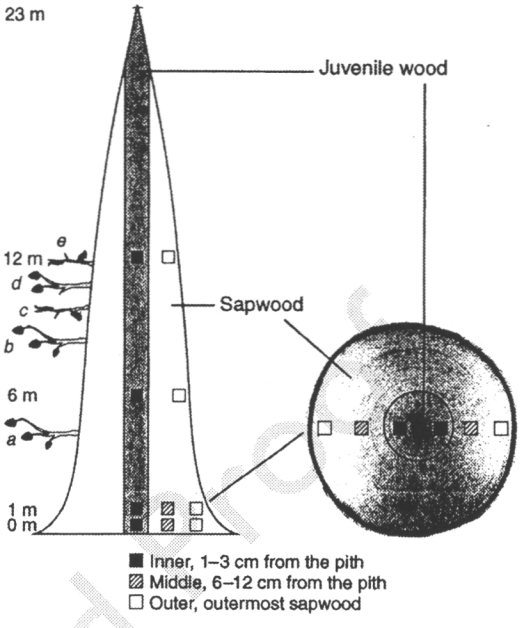

Figure 1. Sampling of mature trees. Samples were taken from the inner, middle and outer parts of the wood at stem heights of 0, 1, 6 and 12 m in mature trees with a total mean height of 23 m. Mean growth ring width (± SE) at a stem height of 1 m for Trees 1–5 was 4.36 ± 0.26, 1.56 ± 0.09, 2.96 ± 0.24, 1.56 ± 0.05 and 1.88 ± 0.09 mm, respectively.

from the pith on either side of the stem samples sampled at heights of 0, 1, 6 and 12 m. Additionally, subsamples were also taken at 6–12 cm from the pith (1–3 cm from the cambium) on either side of the stem samples sampled at heights of 0 and 1 m (Figure 1). At each stem height and cross-sectional location, the 2 subsamples taken from different sides of the pith were combined to form a single sample.

Wood samples were cut into pieces, lyophilized (−60 °C for 4 days) and homogenized to a fine powder (Polymix mill, Kinematica AG, Littau-Lucerne, Switzerland) at −30 °C and stored at −20 °C.

Extraction, purification and hydrolysis of lipids

Wood powder (100 mg) was extracted in a mini-Soxhlet apparatus with 20 ml of acetone for 6 h at 56.2 °C. Internal standards (triheptadecanoin, heptadecanoic acid, cholesterol and cholesteryl heptadecanoate) were added to the extraction solvent. The extracts were redissolved in diethyl ether and stored under nitrogen at −20 °C. Free fatty acids (free FA), TG, free sterols (ST) and steryl esters (SE) were separated by thin-layer chromatography (TLC, Merck 1.05715, 0.20 mm silica gel). The plates were developed in 85:15:1 (v/v) petroleum ether (boiling point 40–60 °C):diethyl ether:acetic acid (Ekman 1979, Saranpää and Nyberg 1987*a*, Kates 1988). The fractions were located under UV light after spraying the edges of the

plates with 0.001% primulin (Sigma P–7522, Wright 1971). The corresponding untreated zones were scraped off and extracted four times in 1 ml of ethyl acetate. The free FA fraction may contain some fatty acids from monoacyl- and diacylglycerol, because they are not completely separated by TLC.

The free FA and TG fractions were saponified with 0.5 M KOH in 90% ethanol for 1 h and the SE fraction was saponified for 3 h at 70 °C. The solutions were diluted with water (1:1 v/v) and acidified to pH 2.0 with 1 M HCl (Ekman 1979). The saponification residues were extracted three times in 1:1 (v/v) *n*-hexane:diethyl ether. Samples were evaporated to dryness in a stream of nitrogen.

Preparation of fatty acid methyl esters (FAME)

Fatty acid methyl esters (FAME) were prepared from the saponified residues by addition of 1 ml of boron trichloride in methanol (Sigma B-1002) at 90 °C for 30 min. Four ml of chloroform and 1 ml of distilled water were added and the samples were shaken vigorously. The upper layer was discarded and the organic phase was then washed three times with 2 ml of Folch 2-phase (Folch et al. 1957). Samples were redissolved in 200 µl of hexane and subjected to gas chromatography (GC) (Nyberg and Koskimies-Soininen 1984).

Preparation of TMS-derivatives of sterol fractions

Samples were evaporated to dryness. Forty µl of water-free pyridin (stored with KOH crystals), 300 µl of bis-trimethylsilyltrifluoroacetamide and 100 µl of trimethylchlorsilane were added to the sample tubes. Samples were incubated for 1 h at 70 °C and stored at −80 °C until analyzed by GC.

Gas chromatography of FAMEs and TMS-esters of sterols

Quantitative measurements of FAME and TMS-esters of sterols were made with a Hewlett Packard gas chromatograph 5890 Series II (Wilmington, DE) with a fire ionization detector. The GC was equipped with a 30 m HP-5 (5% phenyl methyl siloxane, Hewlett Packard, Palo Alto, CA) column with an internal diameter of 0.25 mm and a film thickness of 0.25 µm. For FAME, the column temperature program started at 170 °C and the temperature was increased at 3 °C min^{-1} to a final temperature of 280 °C, which was held for 1 min. For TMS-esters of sterols, the column temperature program started at 110 °C and the temperature was increased at 10 °C min^{-1} to a final temperature of 300 °C, which was held for 40 min.

Helium with an inlet pressure of 130 kPa served as the carrier gas. Split-injection mode was used. The injection volume was 1 µl and the split flow was 20 ml min^{-1} (split ratio 1:10). The septum purge was 2 ml min^{-1}. The injector temperature was 260 °C and the detector temperature was 300 °C. Chromatograms were analyzed with an HP ChemStation program (Agilent Technologies, Wilmington, DE). The FAME and TMS-derivatives were identified by co-chromatography of authentic standard compounds and by GC–MS (gas-chromatography–mass spectrometry) (HP 6890 GC system (Hewlett Packard, Waldbronn, Germany) with an HP 5873 mass selective detector at 70 eV (Hewlett Packard) and temperature program, GC column and gas flow adjustments were made as previously described and by using Wiley 275 GC–MS information). Total ion chromatograms (TIC) of FAME-derivatives and TMS-esters were analyzed. The mass range was 50–600 ion mass units. The identification TMS-esters of sterols was further confirmed by comparing our MS results to isoprenoid mass spectra published for silver birch Kraft black liquor (Niemelä 1990). All lipid concentrations are expressed as µg mg^{-1} of wood dry mass.

Statistical analysis

Statistical analysis was carried out with SPSS Version 9.0.1, (SPSS, Chicago, IL). Gauss distribution of populations was checked by the Kolmogorow-Smirnov test with Lilliefors significance correction. Homogeneity of variance was determined by Levene's test. Lipid concentrations in the seasonal study followed Gauss distribution. Data were evaluated by

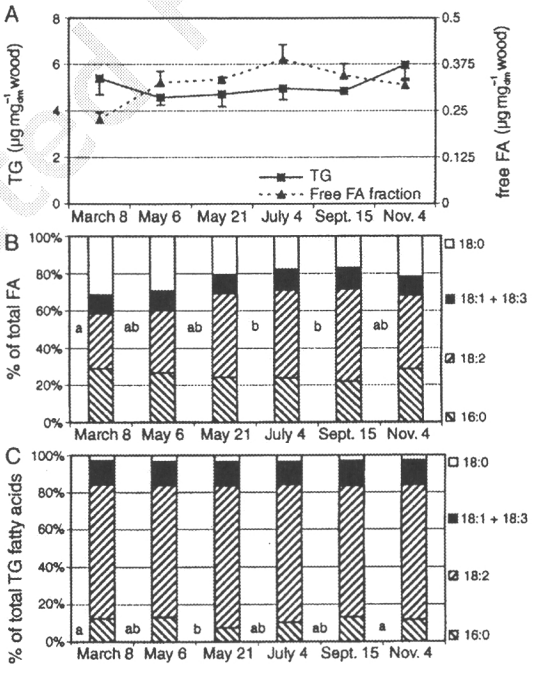

Figure 2. (A) Seasonal changes in concentrations of fatty acid methyl esters (FAME) in the free fatty acid (FA) and triacylglycerol (TG) fractions. Each value represents the mean total concentration of FAME in both fractions of five trees. Bars indicate tree-to-tree variation (standard error; $n = 5$). Bud break occurred at the beginning of May. Yellowing of leaves began in mid-September. Each column represents the mean relative proportions of: stearic acid (18:0); oleic (18:1) and linolenic (18:3) acid; linoleic acid (18:2); and palmitic acid (16:0), in fraction FA (B) and fraction TG (C) of five trees ($n = 5$). Similar letters beside equivalent proportions of each component are not significantly different at $P = 0.01$ for Tukey's test. Letters are not presented when one-way ANOVA indicated no significant difference in concentrations between sampling dates.

1 Only two levels of the

one-way ANOVA, or the Kruskal-Wallis analysis if the variances were unequal (in the case of free linoleic, palmitic and stearic acid, free β-sitostanol and esterified squalene). Both one-way ANOVA and Kruskall-Wallis analysis gave similar results for all dependent variables tested. Pairwise differences were analyzed by the Tukey test.

Lipid concentrations in the within-stem study also followed Gauss distribution and variance was equal except for of squalene (in steryl ester fraction) and β-sitosterol (in free sterol fraction). Data were analyzed by the General Linear Model, which is composed of repeated-measures ANOVA including Huynh-Feldt's test. In the ANOVA model, two internal subject factors and their interaction were tested. Internal subject factors were height of the samples (four levels: 0, 1, 6 and 12 m) and the distance of the sample from the pith (2 levels: 1–3 cm from the pith, and 1–3 cm from the cambium). The distance factor could be tested ~~only at heights of 0 and 1 m~~, because stem diameters at heights of 6 and 12 m were too small to obtain a sufficient number of samples at various distances from the pith for statistical analyses.

Results

In silver birch stem wood, the free FA fractions contained mainly palmitic (hexadecanoic acid, 16:0), linoleic (octadeca-9,12-dienoic acid, 18:2), oleic (9-octadecanoic acid, 18:1), linolenic (octadeca-9,12,15-trienoic acid, 18:3), stearic (octadecanoic acid, 18:0), arachidic (eicosanoic acid, 20:0), behenic (docosanoic acid, 22:0) and lignoceric acid (tetracosanoic acid, 24:0) (Figure 2). Because only trace amounts (< 0.001 μg mg^{-1}) of long-chain saturated fatty acids (20:0, 22:0, 24:0) were detected in young trees, these FA values are not presented in Figure 2. Oleic (18:1) and linolenic acid (18:3) could not be separated by the HP-5 column and were analyzed as one component. Unlike other studies (e.g., Ekman and Pensar 1973), we were unable to detect eicosenoic acid (20:1) based on GC with an HP-5 column.

The ST fraction comprised β-sitosterol, β-sitostanol and campesterol (Table 1). The SE fraction was composed of the isoprenoids: β-sitosterol, β-sitostanol, campesterol, citrostadienol, cycloartenol, 24-methylenecycloartanol, squalene, betulaprenol-6, betulaprenol-7 and betulaprenol-8 (Table 1). Betulaprenol-9 (3,7,11,15,19,23,27,31,35-nonamethyl-2,6,10,14,18,22,26,30,34-hexatriacontanonaen-1-ol) could not be detected by GC with an HP-5 column. In the SE fractions, only trace amounts (< 0.01 μg mg^{-1}) of lupeol were detected in two of the mature stems and so this component is not presented in Figures 3 and 4.

Seasonal variation in lipids in wood of 7-year-old trees

Mean free FA concentration (± SE) was 0.32 ± 0.02 μg mg^{-1}. Free FA concentration was highest during midsummer ($F = 4.27$, $P = 0.006$ in one-way ANOVA, Figure 2A). The average proportion of linoleic acid (18:2), the principal fatty acid in the free FA fraction, was 40.8 ± 3.3% of the total free FA concentration. The concentration of linoleic acid in the FA fraction increased in spring and was highest during midsummer and September ($F = 6.02$, $P = 0.001$, Figure 2B). The mean TG concentration was 5.08 ± 0.21 μg mg^{-1}. The average proportion of the principal fatty acid, linoleic acid, in the TG

Table 1. Mean concentrations of the free sterol and esterified sterol/isoprenoid fractions in silver birch stem wood (μg mg^{-1}). Standard deviations are in parenthesis.

Trivial name of the compound	Chemical name of the compound	Mean concentration for young trees	Mean concentration for mature trees
Free sterols			
β–Sitosterol	(3β)-Stigmast-5-en-3ol	0.154 (0.028)	0.100 (0.034)
β–Sitostanol [1]	(3β,5α)-Stigmastan-3-ol	0.030 (0.006)	0.014 (0.007)
Campesterol	(3β,24R)-Ergost-5-en-3-ol	0.003 (0.002)	0.003 (0.002)
Esterified compounds			
β–Sitosterol	(3β)-Stigmast-5-en-3ol	0.716 (0.162)	0.657 (0.235)
β–Sitostanol [1]	(3β,5α)-Stigmastan-3-ol	0.303 (0.050)	0.202 (0.088)
Campesterol	(3β,24R)-Egost-5-en-3-ol	0.041 (0.025)	0.023 (0.013)
Citrostadienol [2]	(3β,4α,5α,24Z)-4-Methylstigmasta-7,24(28)-dien-3-ol	0.322 (0.072)	0.464 (0.217)
Cycloartenol	Cycloartenol	0.189 (0.156)	0.102 (0.115)
24-Methylenecycloartanol	24-Methylenecycloartanol	0.343 (0.101)	0.243 (0.101)
Squalene	Squalene	0.332 (0.226)	0.619 (0.293)
Betulaprenol-6	3,7,11,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaen-1-ol	0.027 (0.014)	0.055 (0.029)
Betulaprenol-7	3,7,11,15,19,23,27-Heptamethyl-2,6,10,14,18,22,26-octacosaheptaen-1-ol	0.548 (0.154)	1.514 (0.457)
Betulaprenol-8	3,7,11,15,19,23,27,31-Octamethyl-2,6,10,14,18,22,26,30-dotriacontaoctaen-1-ol	0.185 (0.100)	0.982 (0.592)
Lupeol	(3β)-Lup-20(29)-en-3-ol	–	Trace

[1] Also known as stigmastanol.
[2] Also known as α-sitosterol.

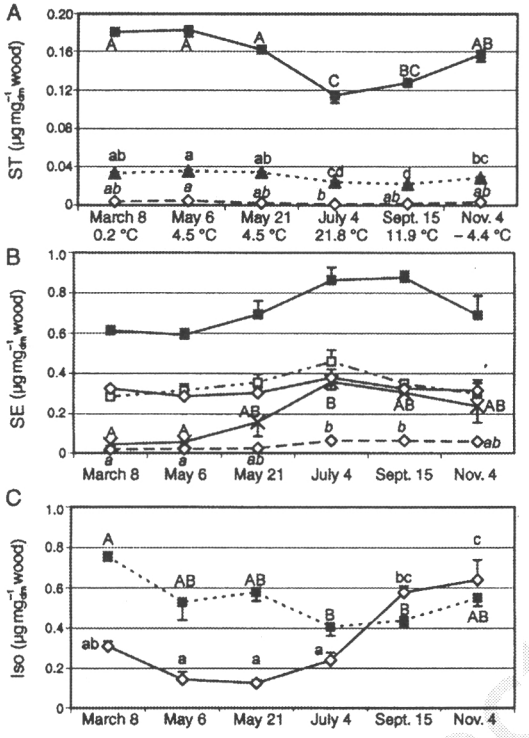

Figure 3. Seasonal changes in concentrations of (A) free sterols (ST) including: β-sitosterol (■); β-sitostanol (▲); and campesterol (◇), (B) steryl esters (SE) including: β-sitosterol (■); campesterol (◇, dashed line); cycloartenol (×); 24-methylenecycloartanol (□); and citrostadienol (◇, solid line), and (C) other SE fraction isoprenoids (Iso) including: squalene (◇); and betulaprenol-7 (■). Each point represents the average sterol/isoprenoid concentration (n = 5 trees); bars indicate standard error. Similar letters beside values of each component are not significantly different at P = 0.01 for Tukey's test. Letters are absent when one-way ANOVA or the following Tukey's test indicated no significant difference in the concentrations between sampling dates. The values below the x-axis of (A) denote the temperature on the corresponding sampling date.

fraction was 72.6 ± 0.9% of the total TG fatty acid concentration. The TG concentration was slightly, although not significantly, higher during the dormant period in November and in March, than during summer and spring (Figure 2A). The palmitic acid (16:0) concentration in the TG fraction was lower in samples taken 2 weeks after bud break in May than in samples taken in March and November (F = 5.12, P = 0.002, Figure 2C).

Mean concentration of ST was 0.19 ± 0.01 μg mg^{-1}. Free β-sitosterol comprised 82.5 ± 0.4% of the total free sterol concentration, whereas the proportion of β-sitostanol was 16.0 ± 0.4%. The concentration of free β-sitosterol markedly decreased toward midsummer, when the temperature was highest, and increased toward November, when the temperature was lowest (F = 22.80, P < 0.001, Figure 3A, for detailed meteorological data, see Piispanen and Saranpää 2001). The free β-sitostanol concentration was highest during bud break in May and lowest when the leaves were yellowing in September (F = 24.68, P < 0.001, Figure 3A). The free campesterol concentration was highest during bud break and lowest during radial growth in July (F = 5.69, P = 0.001, Figure 3A).

The mean total SE concentration was 3.03 ± 0.17 μg mg^{-1}. Seasonal changes were detected in the concentration of SE-bound β-sitosterol, and highest concentrations were measured in July and September (F = 4.08, P = 0.009, Figure 3B). The concentrations of SE-bound cycloartenol (F = 6.76, P = 0.001) and campesterol (F = 8.70, P < 0.001) were greatest during mid-summer in July and lowest during spring from March until bud break in May (Figure 3B). The concentration of squalene was greatest in samples taken during autumn in September and November (F = 18.87, P = 0.001, Figure 3C). The concentration of betulaprenol-7 decreased during spring reaching a minimum in July (F = 5.85, P = 0.001, Figure 3C).

Within-stem variation in lipids in wood of 35- and 70-year-old trees

The mean free FA concentration (sum of total free FA in the FA fraction) was 0.34 ± 0.02 μg mg^{-1}. The dominant fatty acid in the FA fraction was linoleic acid (54.4 ± 2.1% of total free FA fraction, Figures 5C, 5E and 5G). The TG concentration (total concentration of TG fatty acids) was highest in samples close to the pith (Figure 4B, Table 2). Mean TG concentrations in the inner and outer parts of the stem were 9.07 ± 1.19 and 4.50 ± 0.45 μg mg^{-1}, respectively. The dominant fatty acid in TG fraction was linoleic acid (78.8 ± 0.6% of total TG fatty acids). The concentrations of saturated fatty acids (16:0, 18:0, 20:0, 22:0 and 24:0) and linoleic acid (18:2) were higher in samples close to the pith than in samples close to the cambium (Figures 5D, 5F and 5H, Table 2).

Free β-sitosterol and free β-sitostanol comprised about 85.8 ± 0.4 and 12.0 ± 0.5%, respectively, of the total ST concentration. Concentrations of free β-sitosterol and free β-sitostanol were higher in samples close to pith than in samples close to the cambium (Figures 4A and 4B, Table 3). Concentrations of steryl/isoprenoid esters in the inner and outer stem parts were 5.94 ± 0.31 and 4.04 ± 0.33 μg mg^{-1}, respectively. In the SE fraction, the concentrations of campesterol, citrostadienol, 24-methylenecycloartanol, squalene and betulaprenol-6 were significantly higher in samples close to pith than in samples close to the cambium (Figures 4C and 4F–I, Table 3).

Discussion

In young and mature stem wood of silver birch, free FA comprised less than 5% of the total concentration of TG (Figures 2A, 5A and 5B). In Scots pine sapwood, the free fatty acid component constitutes about 10% of the total TG fraction (Saranpää and Nyberg 1987*b*, Fischer and Höll 1992). In stem wood of both young and mature silver birch trees, the concentrations of free FA and linoleic acid in the FA fraction were highest during midsummer (Figures 2A, 2B, 5A, 5C, 5E and

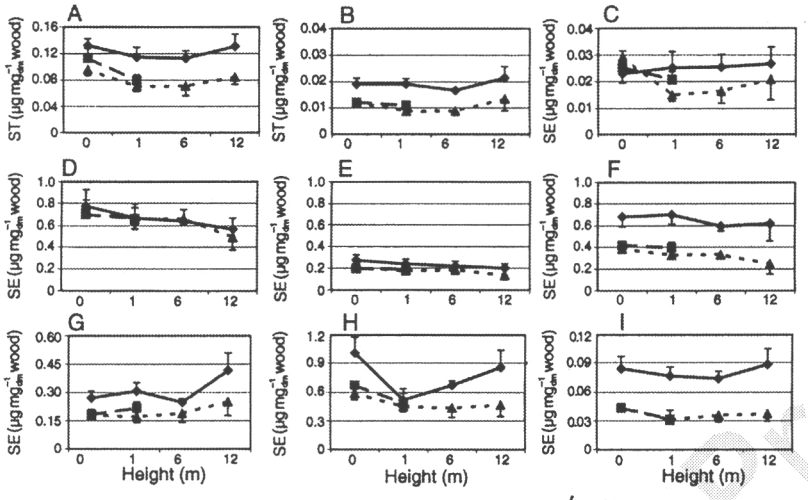

Figure 4. Partitioning of free sterols (ST): (A) β-sitosterol; and (B) β-sitostanol and stearyl esters (SE): (C) campesterol; (D) β-sitosterol; (E) β-sitostanol; (F) citrostadienol; (G) 24-methylenecycloartanol; (H) squalene; and (I) esterified betulaprenol-6 in the stem. Each value represents the mean sterol concentration of five trees (except in G, where $n = 3$). Error bars indicate standard error. Samples were taken 1–3 cm (inner; ◆) or 3–6 cm (middle; ▲) from the pith, and 1–3 cm from the cambium (outer; ■). For statistics, see Table 3. Abbreviation: dm = dry mass.

5G), and the ~~fatty acid component of the~~ FA fraction had a larger proportion of saturated acids (palmitic acid and stearic acid) than the TG ~~component~~. Similarly, in Scots pine sapwood, free FA were more saturated during winter than during summer and were also more saturated than the TG fatty acids (Saranpää and Nyberg 1987*b*). These authors concluded that changes in the composition of the FA fraction reflect the metabolic activity of the Scots pine trees, with the wood parenchyma cells having more saturated free FA during the winter rest period than at other times of the year (Saranpää and Nyberg 1987*b*).

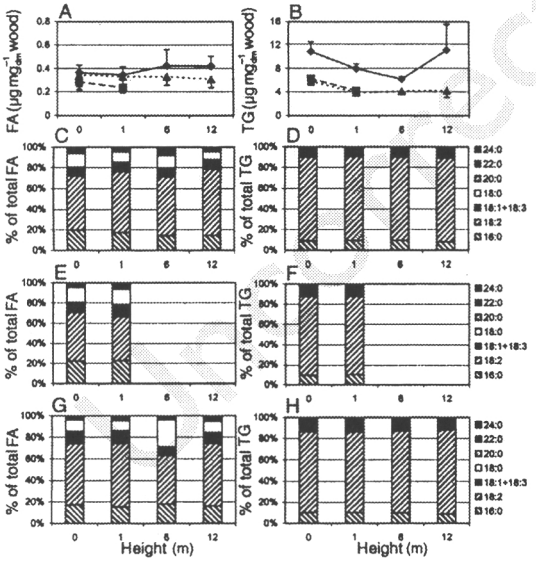

Figure 5. Partitioning of (A) free fatty acids (FA) and (B) triacylglycerols (TG) within the mature stem. Each value represents the mean total lipid concentration (the sum of measured fatty acid methyl esters) of five trees. Bars indicate standard error ($n = 5$). Each column represents the mean relative proportions of: lignoceric acid (24:0); behenic acid (22:0); arachidic acid (20:0); stearic acid (18:0); oleic (18:1) and linolenic (18:3) acid; linoleic acid (18:2); and palmitic acid (16:0), in free FA (C, E and G) and TG (D, F and H) fractions of five trees ($n = 5$). Samples were taken 1–3 cm from the pith (C and D, ◆ in A and B), 3–6 cm from the pith (E and F, ▲ in A and B) and 1–3 cm from the cambium (G and H, ■ in A and B). For statistics, see Table 2.

In stem wood of our young trees, the composition of TG fatty acids remained stable throughout the year, except for a slight decrease in palmitic acid during May (Figure 2), in agreement with observations made in Scots pine sapwood (Saranpää and Nyberg 1987*b*). Both the total concentration and the seasonal variation of storage lipids (TG) were low in our young trees. Silver birch is a diffuse-porous angiosperm, so according to the definition of Sinnot (1918) it is classified as a fat-storing tree like linden (*Tilia cordata* Mill.), which has been reported to show large seasonal changes in the TG concentrations (Höll 1975, Höll and Priebe 1985). However, as in our study, Hoch et al. (2003) did not find significant seasonal variation in storage lipids in sapwood of several coniferous and deciduous trees, including some diffuse-porous angiosperms.

Piispanen and Saranpää (2001) reported a significant increase in starch concentration in stem wood of young silver birch during fall. We analyzed the same samples and found no significant changes in TG concentration (Figure 2a, Table 2). Similarly, no significant conversion of starch to fat was detected during the autumn in samples collected from 6- to 8-year-old branch wood of silver birch (Harms and Sauter 1992), branch wood of poplar (*Populus* × *canadensis* Moench '*robusta*', Sauter and van Cleve 1991, 1994) or stem wood of Norway spruce (Höll 1985). Harms and Sauter (1992), who studied branch wood of silver birch, reported lower TG concentrations (1.25–2.5 μg mg^{-1}) than those we measured in our 7-year-old silver birch stems. These authors detected increases

Table 2. Repeated measures ANOVA (Huynh-Feldt's test) for free fatty acid (FA) (total, 16:0, 18:2, 18:1 + 18:3, 18:0, 20:0 + 22:0 + 24:0) and triglycerol (TG) (total, 16:0, 18:2, 18:1 + 18:3, 18:0, 20:0 + 22:0 + 24:0) fractions in mature silver birch trees felled on July 4. The *F*-values are presented for within-subject factors and their interaction with levels of significance indicated (** = $P < 0.01$; and * = $P < 0.05$). For the df- and *F*-values of 20:0 + 22:0 + 24:0, the sum of these FA methyl esters was used. Total indicates the sum of the measured FAMEs (16:0, 18:2, 18:1 + 18:3, 18:0, 20:0, 22:0, 24:0).

	Height		Distance		Distance × height	
	df	*F*	df	*F*	df	*F*
FA, total	2.08	0.14	1.00	1.64	3.00	0.43
FA, 16:0	1.78	1.10	1.00	3.88	1.13	0.29
FA, 18:2	2.95	0.33	1.00	1.54	3.00	0.69
FA, 18:1 + 18:3	2.54	0.23	1.00	< 0.01	3.00	0.92
FA 18:0	1.05	0.79	1.00	1.06	1.05	0.81
FA, 20:0 + 22:0 + 24:0	1.46	0.58	1.00	1.86	1.08	1.22
TG, total	1.19	1.41	1.00	20.95 *	1.80	0.70
TG, 16:0	1.18	1.51	1.00	37.00 **	1.57	0.71
TG, 18:2	1.22	1.41	1.00	21.07 *	1.85	0.69
TG, 18:1 + 18:3	1.15	1.60	1.00	5.77	1.55	0.87
TG, 18:0	1.45	1.21	1.00	23.99 **	1.38	0.66
TG, 20:0 + 22:0 + 24:0	2.6	1.38	1.00	18.45 *	1.53	0.49

in two peaks in the storage fat profile during leaf yellowing and leaf fall (Harms and Sauter 1992), whereas we observed that only a single TG fatty acid (palmitic acid) increased when our 7-year-old trees were dormant.

Within-stem variation in the concentration of TG-bound fatty acids was larger in mature trees than in young trees during midsummer (Figures 2A and 5B), probably because the young trees belonged to the same clone. In mature silver birches, the TG concentration was higher in samples close to the pith than in samples close to the cambium (Figure 5B, Table 2). Similarly, Höll and Poschenrieder (1975) found that the TG concentration increases toward the pith. Because silver birch does not form heartwood (Piispanen and Saranpää 2001), the storage lipids seem to accumulate in the living parenchyma cells close to the pith in mature trees.

In our trees, the free sterol fraction comprised mainly β-sitosterol (Figures 3A, 4A and 4B, Table 1). Similar compositions have been reported for stem wood of Scots pine (Saranpää and Nyberg 1987*a*) and black locust (Hillinger et al. 1996). As in other studies (Selleby 1960, Assarsson and Åkerlund 1966), the steryl ester fraction was composed of a wide range of major STs and their intermediates, including β-sitosterol, citrostadienol, cycloartenol and 24-methylenecycloartanol, and other isoprenoids typical of silver birch wood such as squalene and betulaprenols-7 and -8 (Figure 4, Table 1).

Changes in the concentration of free β-sitosterol were directly associated with changes in air temperature (Figure 3A). In plant cells, free ST accumulate in the plasma membrane where they regulate membrane fluidity (Rodriguez et al. 1985). An increase in β-sitosterol concentration in plasma membranes has been detected during cold acclimation of *Solanum* species (Palta et al. 1993) and winter rye seedlings (*Secale cereale* L., Uemura and Yoshida 1984, Lynch and Steponkus 1987). In contrast, when roots of green wheat (*Triticum aestivum* var. Monon) were kept at 1 or 10 °C, the total concentration of free ST and β-sitosterol first decreased and then recovered to a value exceeding that of the original concentration (Davis and Finkner 1972).

In our young trees, the concentrations of free campesterol and β-sitosterol decreased and the concentrations of SE-bound campesterol and β-sitosterol increased during radial growth in midsummer (Figures 3A and 3B). It has been suggested that SE in plants are incorporated, in limited amounts, into membranes (Dyas and Goad 1993) or are involved in storage and transport as in mammalian cells (Grunwald 1980) or serve as an interconvertible pool for free ST and FA (Dyas and Goad 1993, Hillinger et al. 1996). In black locust wood, the concentration gradients of SE are inversely correlated with free sterol concentrations in samples taken from the pith to cambium (Hillinger et al. 1996). In tissue cultures of the sterol-overproducing mutant tobacco (*Nicotiana tabacum* L.), it has been shown that ST accumulate as SE in hyaloplasmic bodies (Gondet et al. 1994). Our study of seasonal variation in lipids supports the storage pool theory by showing that the concentrations of campesterol and β-sitosterol were inversely correlated in the free and esterified steryl fractions (Figures 3A and 3B). In addition, the concentrations of β-sitosterol in the free sterol and SE fractions were inversely correlated in the inner parts of large stems at 12 m height (Figures 1, 4A and 4D).

Cycloartenol is a polycyclic intermediate unique to plant sterol biosynthesis (Clouse 2002), and in silver birch wood, this intermediate is present in esterified form as 24-methylenecycloartanol. In young silver birches, the concentration of SE-bound cycloartenol peaked during maximum radial growth in midsummer (Figure 3B), and the seasonal profile for 24-methylenecycloartanol closely followed that of cycloartenol (Figure 3B). The cycloartenyl esters in *Taraxacum officinale* Weber (Westerman and Roddick 1981) and black locust (Hillinger et al. 1996) show a similar positive correlation with temperature.

Table 3. Repeated measures ANOVA results (Huynh-Feldt's test) for compounds in ST and SE fractions in mature silver birch trees felled on July 4. The *F* values are presented for within-subject factors and their interaction. Asterisks indicate level of significance: *** = $P < 0.001$; ** = $P < 0.01$; and * = $P < 0.05$.

	Height		Distance		Distance × Height	
	df	*F*	df	*F*	df	*F*
Free sterol fraction						
β–Sitosterol	1.36	0.82	1.00	412.09 ***	3.00	0.4
β–Sitostanol	3.00	0.35	1.00	36.05 *	3.00	3.71
Campesterol	3.00	1.02	1.00	0.91	3.00	0.22
Steryl ester fraction						
β–Sitosterol	1.43	1.66	1.00	< 0.01	3.00	3.58
β–Sitostanol	1.89	1.82	1.00	5.83	3.00	2.88
Campesterol	1.34	0.24	1.00	11.15 *	1.75	0.33
Citrostadienol	2.54	0.49	1.00	19.22 *	2.39	1.20
Cycloartenol	3.00	0.99	1.00	< 0.01	1.02	0.74
24-Methylenecycloartanol	2.85	3.10	1.00	8.77	2.90	5.09 *
Squalene	1.26	1.76	1.00	11.74 *	3.00	1.43
Betulaprenol-6	2.18	0.69	1.00	94.27 **	1.41	1.28
Betulaprenol-7	3.00	1.02	1.00	10.39	3.00	2.50
Betulaprenol-8	2.47	1.62	1.00	1.15	3.00	1.62

In young silver birches, the concentration of SE-bound squalene increased and the concentration of SE-bound betulaprenol-7 decreased during spring (Figure 3C), perhaps indicating a seasonal metabolic interconversion between these aliphatic isoprenoids. To our knowledge, seasonal variation of these aliphatic components has not previously been studied in xylem.

In mature silver birches, the concentrations of free and SE-bound ST (β-sitosterol, β-sitostanol and citrostadienol) were higher in samples taken close to the pith than in samples taken close to the cambium (Figures 4A, 4B and 4F, Table 3). Accumulation of free ST and SE in the inner parts of stem wood has been documented for young and mature Scots pine wood (Höll and Lipp 1987). In detailed analyses of the different sapwood zones of Scots pine (Saranpää and Nyberg 1987*a*) and black locust (Hillinger et al. 1996), the free sterol concentration decreased toward the heartwood transition zone, which was taken to indicate membrane deterioration. In photoautotrophic cultures of *Chenopodium rubrum*, the free sterol concentration (i.e., sitosterol, stigmasterol and campesterol) decreased and the concentration of oxidized phytosterols increased during aging (Meyer and Spiteller 1997). However, in black locust inner heartwood, the free sterol concentration was fairly high and a slight decrease in the proportion of β-sitosterol was detected toward the pith (Hillinger et al. 1996). In silver birch wood, the proportion of free β-sitosterol did not decrease from cambium to pith at any of the measured heights (Figures 4A and 4B); thus, no sign of wood senescense in the innermost parts of mature wood was detected. However, in mature wood, the free β-sitosterol concentration was high in young parts of the wood (e.g., at a height of 12 m), and in older parts of the wood (e.g., cells close to pith and at stump height) (Figure 4A), which is consistent with the free sterol profile detected in the trunk of black locust by Hillinger et al. (1996).

We observed accumulation of SE-bound citrostadienol, SE-bound cyclic triterpenoid, 24-methylenecycloartanol and SE-bound acyclic terpenoids like squalene and betulaprenol-6 in the inner parts of mature silver birch wood (Figures 4F–I, Table 3), perhaps indicating that, in silver birch wood, these components function as a reservoir for secondary metabolites of the living cells close to pith. The large variety of SE-bound isoprenoids, especially those having an intermediate in the isoprenoid part of the compound (e.g., 24-methylenecycloartanol and squalene), maybe a consequence of the exceptionally high concentration of the SE fraction in silver birch when compared with Scots pine (Höll and Lipp 1987, Saranpää and Nyberg 1987*a*, Saranpää and Piispanen 1994).

In conclusion, we found marked seasonal variation in the concentrations of isoprenoids and one TG fatty acid and in the size of the free fatty acid fraction in silver birch. Large seasonal variation in neutral lipids in stem wood is a characteristic feature of deciduous fat-storing trees like *Tilia cordata* Mill. (Höll and Poschenrieder 1975) that accumulate lipophilic constituents in the inner parts of the stem; however, the seasonal variation in TG concentration in silver birch was small and not typical of a fat-storing tree. Silver birch had high concentrations of steryl and other isoprenoid esters in mature wood and also in the young xylem, indicating a high capacity for isoprenoid formation and acylation. We found no evidence of heartwood formation in silver birch wood.

Acknowledgments

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