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, 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 Koprina 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 Minnchx.) (Tuominen et al. 1995, 1997, Fladung et al. 1996, 1997a, 1997b, Nilsson et al. 1996a, 1996b, 1997, Tzfira et al. 1998), as well as kiwi (Actinidia delicosa 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. 1996b), 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 CS8 (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 aux (auxin biosynthesis), mas (mannopine synthesis), ags (agropine synthesis) and rolB\(^{ma}\) 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 thidiazuron (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
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 \textit{Hin}dIII or \textit{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 \textit{aux1}, \textit{aux2}, \textit{rolA}, \textit{rolB}, \textit{rolC} and \textit{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 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.

**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.**

<table>
<thead>
<tr>
<th>Primer sequences 5′-3′</th>
<th>Amplified fragment/probe</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTGCCTCTAAATAGCGACCG</td>
<td>468-bp Fragment; nucleotides 112–581(^1) of the trans-zeatin secretion (\textit{tzs}) gene of the pRiA4 virulence region</td>
<td>PCR screening</td>
</tr>
<tr>
<td>CTGGCAACTGTTGATTGCCG</td>
<td>791-bp Fragment of \textit{aux1}; nucleotides 3787–4578(^2) from the pRiA4 TR-DNA</td>
<td>PCR screening Northern</td>
</tr>
<tr>
<td>CTCCGATTCCTTCAACCG</td>
<td>516-bp Fragment of \textit{aux1}; nucleotides 4918–5434(^3) from the pRiA4 TR-DNA</td>
<td>Northern</td>
</tr>
<tr>
<td>CGCAATTTCTCTTATACC</td>
<td>722-bp Fragment of \textit{aux2}; nucleotides 1693–2415(^3) from the pRiA4 TR-DNA</td>
<td>PCR screening Southern Northern</td>
</tr>
<tr>
<td>ACGGTGAGTGGTTTAGG</td>
<td>403-bp Fragment of \textit{rolA}; nucleotides 9656–10,059(^3) from the pRiA4 TL-DNA</td>
<td>PCR screening Southern Northern</td>
</tr>
<tr>
<td>GCCACGTGCATTTAATCCC</td>
<td>696-bp Fragment of \textit{rolB}; nucleotides 10,520–11,216(^3) from the pRiA4 TL-DNA</td>
<td>PCR screening Southern Northern</td>
</tr>
<tr>
<td>TGGACAACGAGCGATGAC</td>
<td>480-bp Fragment of \textit{rolC}; nucleotides 12,513–12,993(^3) from the pRiA4 TL-DNA</td>
<td>PCR screening Southern Northern</td>
</tr>
<tr>
<td>AAACCTGGACTCCGCAAG</td>
<td>741-bp Fragment of \textit{rolD}; nucleotides 16,634–17,375(^3) from the pRiA4 TL-DNA</td>
<td>PCR screening Southern</td>
</tr>
<tr>
<td>ATGCACTGTAAGGAGCACCTC</td>
<td>477-bp Fragment of \textit{rolD}; nucleotides 17,053–17,530(^3) from the pRiA4 TL-DNA</td>
<td>Northern</td>
</tr>
<tr>
<td>GCCACGTGCGTATTAATCCC</td>
<td>493-bp Fragment of birch ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (\textit{RbcS}) gene, nucleotides 210–703 of genomic clone</td>
<td>PCR screening</td>
</tr>
</tbody>
</table>

1 According to Regier et al. (1989).
3 According to Slightom et al. (1986).

**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 \textit{aux1}, \textit{aux2}, \textit{rolA}, \textit{rolB}, \textit{rolC} and \textit{rolD} are described in Table 1. After hybridization, membranes were washed twice for 5 min with 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 phytotormone-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.

**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, Kinematika 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 Klassen 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 Klassen 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 absorbivity of 110 l g–1 cm–1.

A sample of 5 ml of the filtrate was neutralized with BaCO3 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, 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/camb-

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**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).**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group I¹</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of plants</td>
<td>31</td>
<td>7</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>Hairy root clone that plants originated from</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Stem height (cm)</td>
<td>153 ± 9 a</td>
<td>88 ± 9 b</td>
<td>57 ± 7 b</td>
<td>140 ± 10 a</td>
</tr>
<tr>
<td>Stem diameter (mm)</td>
<td>12.1 ± 0.3</td>
<td>13.0 ± 0.9</td>
<td>12.5 ± 0.5</td>
<td>12.1 ± 0.5</td>
</tr>
<tr>
<td>Roots (percent of total fresh mass)</td>
<td>15.8 ± 1.4 a</td>
<td>28.6 ± 2.8 b</td>
<td>22.7 ± 1.7 b</td>
<td>16.4 ± 2.7 a</td>
</tr>
<tr>
<td>Leaf size (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Top leaves</td>
<td>73 ± 3 a</td>
<td>33 ± 2 b</td>
<td>35 ± 3 b</td>
<td>69 ± 3 a</td>
</tr>
<tr>
<td>Central leaves</td>
<td>49 ± 2 a</td>
<td>29 ± 3 b</td>
<td>28 ± 2 b</td>
<td>49 ± 2 a</td>
</tr>
<tr>
<td>Basal leaves</td>
<td>38 ± 1 a</td>
<td>24 ± 2 b</td>
<td>23 ± 2 b</td>
<td>37 ± 2 a</td>
</tr>
<tr>
<td>Percentage of plants with single stem</td>
<td>68</td>
<td>43</td>
<td>17</td>
<td>35</td>
</tr>
<tr>
<td>Xylem diameter</td>
<td>8.5 ± 0.2 a</td>
<td>6.3 ± 0.5 b</td>
<td>4.9 ± 0.6 c</td>
<td>7.6 ± 0.2 ab</td>
</tr>
<tr>
<td>Mean growth ring width</td>
<td>0.98 ± 0.05 a</td>
<td>0.79 ± 0.07 bc</td>
<td>0.56 ± 0.03 c</td>
<td>0.95 ± 0.02 a</td>
</tr>
</tbody>
</table>

¹ Plants were decapitated during the fourth growing season.
² Data from 12 individual plants.
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 7b).
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 root tissues 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,
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. 1996a), 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. 1996a). In transgenic hybrid aspen, the rolC promoter was activated by sucrose (Nilsson et al. 1996a). 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 (Nilsson and Olsson 1997).

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 (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
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. 1996b, 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. 1996b). Expression of 35S-rolC was detected in leaves of transgenic hybrid aspen (Nilsson et al. 1996b), 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 1996b, Grundwald 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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