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ORIGINAL ARTICLE

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Does lignin modification affect feeding preference or growth performance of insect herbivores in transgenic silver birch (*Betula pendula* Roth)?

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Abstract Transgenic silver birch (*Betula pendula* Roth) lines were produced in order to modify lignin biosynthesis. These lines carry *COMT* (caffeate/5-hydroxyferulate *O*-methyltransferase) gene from *Populus tremuloides* driven by constitutive promoter 35S CaMV (cauliflower mosaic virus) or UbB1 (ubiquitin promoter from sunflower). The decreased syringyl/guaiacyl (S/G) ratio was found in stem and leaf lignin of 35S CaMV-PtCOMT transgenic silver birch lines when compared to non-transformed control or UbB1–PtCOMT lines. In controlled feeding experiments the leaves of transgenic birch lines as well as controls were fed to insect herbivores common in boreal environment, i.e., larvae of *Aethalura punctulata*, *Cleora cinctaria* and *Trichopteryx carpinata* (Lepidoptera: Geometridae) as well as the adults of birch leaf-feeding beetles *Agelastica alni* (Coleoptera: Chrysomelidae) and *Phyllobius* spp. (Coleoptera: Curculionidae). The feeding preferences of these herbivores differed in some cases among the tested birch lines, but these differences could not be directly associated to lignin modification. They could as well be explained by other characteristics of leaves, either natural or caused by transgene site effects. Growth performance of lepidopteran larvae fed on transgenic or control leaves did not differ significantly.

Keywords *Betula* · Insect herbivores · Lignin modification · *O*-methyltransferase · Syringyl/guaiacyl ratio

Abbreviations *COMT*: Caffeate/5-hydroxyferulate *O*-methyltransferase · *S*/*G*: Syringyl/guaiacyl

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Introduction

Lignin is a phenolic heteropolymer that is deposited together with polysaccharides in plant cell walls, and has the strengthening and water transportation function. Biosynthesis and properties of wood lignin have been studied extensively (Sarkanen 1971; Higuchi 1985) and during the last decade, studies on genetically-modified (GM) trees have gained more information on the lignin biosynthetic pathway (Baucher et al. 1998; Hu et al. 1999; Anterola and Lewis 2002; Boerjan et al. 2003). When considering practical applications, the lignin-modified wood may turn out to be a potential raw material for the pulp industry, if the modified lignin properties can be related to improved delignification (pulping) characteristics compared to natural raw material. Alternatively, lignin modification could also lead to high-energy wood production.

Wood lignin modifications have, however, evoked concern about the potential effects on tree fitness and also of potential unintended side effects and pleiotrophic effects of the transgenes especially when constitutive promoters are used (James et al. 1998; Campbell and Asante-Owusu 2001). A large-scale production of superior GM-clones as field growing cultivars is required for industrial purposes. Before practical cultivation is undertaken, the potential ecological impact of GM trees

needs to be investigated. This is especially important because forest trees are characterised by a long life cycle, by wind pollination in most cases and by diverse biotic interactions. So far the experience on the field-grown lignin-modified forest trees is limited (Pilate et al. 2002).

Silver birch (*Betula pendula* Roth) is economically the most important deciduous tree species in Nordic countries. In Finland, approximately 15% of growing stock (311 million m^3) is birch (Peltola 2003), and the birch roundwood is raw material in the chemical pulp industry. Silver birch is also the main broad-leaf species of conventional tree breeding in Finland (Haapanen and Mikola 2004). In addition, silver birch is applicable to molecular breeding, due to the existing tissue culture propagation methods (Ryynänen and Ryynänen 1986), genetic transformation techniques (Keinonen-Mettälä et al. 1998; Valjakka et al. 2000), and EST-libraries (Palva 2000) allowing further functional high throughput genome-wide analyses. Silver birch is also one of the key species in Nordic forest ecosystems, and its ecological interactions have been studied widely (Rousi et al. 1997; Mutikainen et al. 2000; Prittinen et al. 2003a, b; Tikkanen et al. 2003; Laitinen et al. 2004). Till date, the experience on the potential ecological impacts of GM birches is limited (Pasonen et al. 2004).

The aim of the current work was to study the interactions between lignin-modified silver birches and their common leaf-consuming insect herbivores. We analysed the leaf lignin in order to see, if the genetic modification had affected lignin content and/or composition. In controlled feeding experiments, the leaves of both lignin-modified trees and control trees were fed to lepidopteran larvae and coleopteran species in order to examine the possible effect of the lignin modification on preference (food selection) or performance [relative growth rate (RGR)] of the herbivores.

Materials and methods

Production of transgenic birches

Two gene constructs, pRT99/35S-*PtCOMT* (6.2 kb) and pRT99/UbB1-*PtCOMT* (6.9 kb; Fig. 1), carrying the complete cDNA of the bispecific caffeate/5-hydroxyferulate *O*-methyltransferase (*COMT*) from *Populus tremuloides* (L.) (Bugos et al. 1991) were prepared for genetic transformation of birch according to Aronen et al. (2003). Genetically modified silver birch (*Betula pendula* Roth) lines were produced by introducing the gene constructs into birch clone A by particle bombardment. Selection and regeneration of transgenic birch lines were performed as described by Aronen et al. (2003).

Greenhouse cultivation of birch lines

Silver birch plants from the 35S-PtCOMT-lines 23 and 44, UbB1-PtCOMT-lines 65 and 130 as well as non-transgenic controls representing clone A were grown under standard greenhouse conditions, and their growth and wood characteristics were analysed as described in Aronen et al. (2003). The plants were decapitated after the second growing season because of the limited greenhouse space. Plants of the line 130 were not decapitated because they followed a belated growth rhythm and started to grow several weeks later than control or other lines. This resulted in a smaller plant size compared to other lines. In the herbivory feeding experiments, the birch lines were represented by either 13 (lines 23, 44, 65 and control) or 7 (line 130) plants.

Molecular analyses

Southern analysis

Total genomic DNA was isolated from the leaves by the modified method of Lodhi et al. (1994) as described in Valjakka et al. (2000) and Aronen et al. (2003). Southern blot analyses were performed to verify the integration of the transgenes into the birch genome. Twenty micrograms of genomic DNA was digested overnight with *Sac*I (having a unique restriction site within the constructs) and fractioned on a 0.8% (w/v) agarose gel at 70 V for approximately 4 h. The gels were depurinated, denaturated, neutralised and the fragmented DNA was transferred to a nylon membrane (Roche Diagnostics

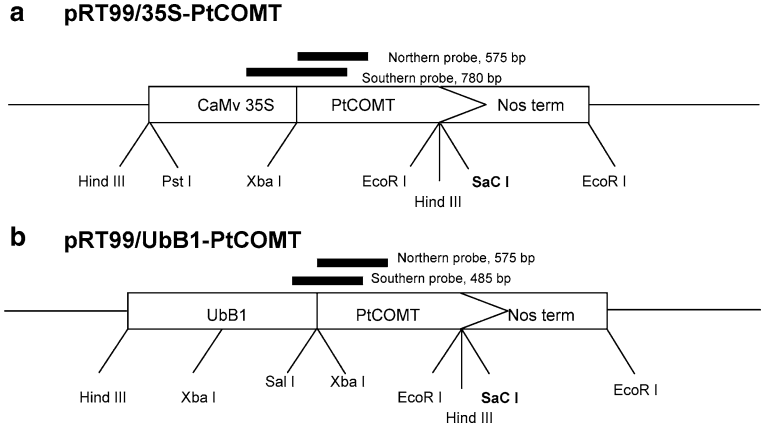

Fig. 1 Schematic presentation of the pRT99/35S-PtCOMT (**a**) and pRT99/UbB1-PtCOMT (**b**) constructs with the localisation of the probes applied in Southern and Northern verification. In Southern analyses, *Sac*I (marked with bolding) restriction site was used for DNA digestion

GmbH, Mannheim, Germany) by capillary transfer. The prehybridisations and hybridisations were performed in a Easy Hyb solution (Roche Diagnostics GmbH) at 42–45°C.

Double stranded probes for *nptII* (0.7 kb), 35S-*COMT* (0.78 kb) and UbB1-*COMT* (0.485 kb) (see Fig. 1) were labelled with digoxigenin-11-dUTP in the PCR using the conditions according to Aronen and Häggman 1995 and the following primers: For *nptII* the sense primer was 5′-TGGGCACAACAGACAATC GG-3′ and the antisense primer was 5′-CAGCAATA TCACGGGTAGCC-3′. The sense primer for the 35S-promoter was 5′-AGTCTCAGAAGACCAGAGGG-3′, the sense primer for the UbB1-promoter was 5′-AACC GGAAAGACCATCACCC-3′ and the antisense primer for *COMT*-gene was 5′- TTACAAACAGGAGCGAG GCC-3′.

After overnight hybridisation, the blots were washed twice with 2×SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) containing 0.1% SDS for 5 min at room temperature and at high stringency twice with 0.5×SSC, 0.1% SDS at 68°C for 15 min. The chemiluminescence detection of hybridisation products was performed according to the manufacturer's (Roche Diagnostics GmbH) instructions.

Northern analysis

Total RNA was isolated from leaves, developing xylem or phloem by the modified method of Chang et al. (1993) and as described by Aronen et al. (2003) during the growing season. Northern blot analyses were performed to study the functioning of the *PtCOMT* transgene in birch tissues. Fifteen micrograms of total RNA was separated on 1.2% agarose gel containing 2.9% formaldehyde following denaturation of samples at 100°C for 2 min in formaldehyde and formamide. The electrophoretically separated RNAs were transferred to a nylon membrane (Roche Diagnostics GmbH) by capillary transfer in 20×SSC overnight. After blotting, the membrane was washed twice in 2×SSC at room temperature for 10 min and crosslinked by UV-illumination. The efficiency of the RNA transfer was determined by staining the membrane in methyleneblue (0.02% w/v methyleneblue, 0.3 M sodium acetate, pH 5.5) for 3 min.

Before hybridisation, the membrane was destained in 0.1×SSC, 0.5% SDS at 68°C for 15 min. The prehybridisation was performed in Easy Hyb Solution at 50°C for 1–2 h. For hybridisation, a fresh Easy Hyb Solution containing denaturated PtCOMT probe for the detection of the corresponding mRNA (with a coding region of 1.1 kb) was used. The PtCOMT probe (0.575 kb) (see Fig. 1) was labelled with digoxigenin-11-dUTP by the PCR using the primers 5′-CAGGTATCAGATGAAG-AGGC-3′ and 5′-AACATCCACCAAGGACGTG-3′. After overnight hybridisation at 50°C, the blot was washed twice with 2×SSC, 0.1% SDS for 5 min at room temperature and at high stringency twice with 0.2×SSC, 0.1% SDS at 68°C for 15 min. The hybridisation products were detected according to the manufacturer's (Roche Diagnostics GmbH) instructions.

Leaf sampling for feeding and lignin experiments

For the feeding experiments, the fully opened leaves including the petioles were collected randomly from the uppermost branches of the 3-year-old plants at the time of the emergence of the tested herbivores. In each individual experiment, the leaf material tested was even-aged. In the repeated feeding experiments, the age of the leaves varied from 4 weeks to 8 weeks. For lignin analyses, 6-week-old leaves without the petioles were collected as in feeding experiments during the fourth growing season. When sampling, the leaves were collected evenly from all the experimental plants representing the individual birch line.

Lignin analyses

Klason and acid-soluble lignin analyses The leaf samples were first dried at 60°C for 2 days and then homogenized to fine powder. The dry mass of the leaves was determined by drying a subsample at 103°C for overnight. Powdered samples of leaves (3 g) were extracted with acetone, ethanol and water for 6 h per solvent in a Soxhlet apparatus, and two parallel extractions were performed per each leaf origin. The Klason lignin content of the leaves was analysed by the modified method of Effland (1977). Five parallel determinations were carried out per sample. Three hundred milligrams of extractive-free leaf powder was hydrolysed in 3 ml of 72% H_2SO_4 for 1 h in an ultrasonication bath. Then 82 ml of ion-exchanged water was added to the samples, and samples were autoclaved at 125°C, 0.1 MPa for 1 h. After cooling, the samples were filtered through fritted crucibles, washed and dried, and the acid-insoluble Klason lignin was determined gravimetrically. The filtrate was diluted to 250 ml and acid-soluble lignin was measured by ultraviolet absorption at 203 nm with spectrophotometer (Shimadzu UV-2401 PC UV-Vis recording spectrophotometer), using a lignin absorptivity of 110 l g^{-1} cm^{-1} . According to Dyckmans et al. (2002) reporting the protein accumulation into the gravimetric lignin of leaf material, the nitrogen content of air-dried Klason lignin samples was analysed by LECO-1000 Elemental analyser.

Analysis of leaf lignin monomers The syringyl/guaiacyl (S/G) of leaf lignin was determined by the modified method of thioacidolysis (Rolando et al. 1992). An extractive-free leaf sample of 10–30 mg was mixed with 3 ml freshly prepared thioacidolysis reagent (2 M BF_3 etherate in a 8.75:1 (v/v) dioxane/ethanethiol mixture). The thioacidolysis proceeded at 103°C for 4 h. After cooling, the reaction mixtures were rinsed with water in the reaction tube containing methylene chloride (CH_2Cl_2). The lignin fragments were extracted from the

aqueous phase thrice with 2 ml CH_2Cl_2. The organic fractions were combined, dried by the addition of anhydrous Na_2SO_4 and evaporated under nitrogen. Before the GC-MS analysis, the dried samples were silylated with 0.5 ml 20% TMSI-pyridine mixture (TMSI = 1-(trimethylsilyl)imidazole) at 60°C for 1 h and then the silylation was continued at room temperature overnight. The GC-MS analyses were performed using a HP 6890 GC-system equipped with mass selective detector 5873 and HP-5 capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness). Helium was used as a carrier gas, with a flow of 1.5 ml/min.

The chromatographic conditions were as follows: initial temperature, 180°C; temperature rate, 5°C/min; final temperature, 280°C for 5 min; injector temperature, 280°C; transfer-line temperature, 300°C; split ratio, 1:20. The mass spectral characterisations were done according to Rolando et al. (1992). The ratio was calculated by using a peak area, for syringyl lignin fragments S-CHR-CHR-CH_2R and S-CH_2-CHR-CHR_2; for guaiacyl lignin fragments G-CHR-CHR-CH_2R and G-CH_2-CHR-CHR_2, where S = syringyl; G = guaiacyl and R = Set, where S = sulphur and et = ethyl.

Herbivore material

Insect herbivores

Herbivores used in experiments were common and polyphagous birch feeding species including moths *Aethalura punctulata* (Denis and Schiffermüller), *Cleora cinctaria* (Denis and Schiffermüller) and *Trichopteryx carpinata* (Borkhausen) (Lepidoptera: Geometridae), and leaf beetles *Agelastica alni* (L.) (Coleoptera: Chrysomelidae) and *Phyllobius* spp. (Coleoptera: Curculionidae). The moths and adult leaf beetles were collected at the time of their occurrence in April–June and June–July 2002, respectively. Insects were collected in Punkaharju, Finland (61°48′N, 29°19′E), except *T. carpinata* was collected from Parikkala, Finland (61°33′N, 29°30′E). One female moth with one male were let to lay eggs on birch twigs in a growing chamber, and when hatched, larvae were let to develop until they reached the size of 1–1.5 cm. The larvae represented families of 4 (*Cleora cinctaria*) to 20 (other geometrids) females. Families were randomised within each species, and middle-sized larvae were selected for experiments. Only active larvae were selected for the experiments. All the lepidopteran larvae, as well as the adults of *A. alni* and *Phyllobius* spp., were fed with fresh birch twigs until used in the experiments.

Experimental design for food selection experiments

The experiments were carried out in Petri dishes (Ø 14 cm). The leaf petiole was put into a 200 μl Eppendorf tube filled with water and the leaf lamina was placed on the moisturised filter paper. Before the feeding experiment, the initial leaf area was scanned with Epson expression 1680 scanner and WinFOLIA Pro (Regent Instruments Inc.) software. One transgenic (23, 44, 65 or 130) and one control (the same birch genotype without gene transferring) leaf were placed on the same dish, and the feeding was started by placing one geometrid larva or beetle between the leaves. The feeding continued for 24 or 48 h depending on the activity of the species.

The feeding experiment with *A. punctulata* contained 15 replicates (= Petri dishes) and that with *C. cinctaria* contained 20 replicates for each of the lines 23, 44, 65 and 130. In the feeding experiment of *T. carpinata*, there were 15 replicates for the lines 23, 44 and 65. The feeding experiments of *A. alni* and *Phyllobius* spp contained 20 replicates for the lines 23, 44 and 65. After the feeding experiment, the final leaf area was scanned and analysed as described above. The leaf consumption was determined as a difference between the initial and final leaf area. The cases with no observable leaf consumption were excluded from the analyses.

Experimental design for relative growth rate (RGR) experiment

The experiments were carried out in Petri dishes (Ø 9 cm), and the collection, handling and scanner analyses of the leaves were done as in food selection studies. The RGR test of *A. punctulata* consisted of 15 replicates for the transgenic lines 23, 44, 65 and control, and 7 replicates for the transgenic line 130. In the RGR experiment of *C. cinctaria*, there were 20 replicates for the transgenic lines 23, 44, 65, 130 and control. In the RGR test of *T. carpinata*, there were 15 replicates for the transgenic lines 23, 44, 65 and control. The weight of larvae was measured, whereupon it was put on a dish containing one transgenic or one control leaf. The feeding was let to continue for 24 or 48 h depending on the feeding activity of the species. The final weight of larvae was measured, and the relative growth rate of larvae was calculated as a proportional weight gain during the experiment. The larvae that were dead or lost weight were excluded from the analyses.

The leaf vein consumption

The leaf vein consumption was determined as a percentage of consumed veins per leaf, and it was issued on both food selection and RGR experiments. The midvein and principal lateral veins were observed, and the untouched leaves were omitted from the analyses.

Statistical analyses

Leaf lignin results were analysed with analysis of variance (arcsin square transformation was used for

quantitative lignin data). The Levene's test was applied for the test of equality of error variances. If equality was demonstrated, the pairwise comparisons were performed with Tukey's HSD test, otherwise the Tamhanes's test was used.

For the analyses of food selection experiments, leaf consumption was first converted into a binary response (preferred or not preferred). To test whether hypothesis concerning preferred leaf origin (transgenic vs. control) could be based on pooled data (combining results from different lines), a test of heterogeneity was applied. If the homogeneity was established, the transgenic leaf versus control leaf-selection was tested with χ^2-test in the pooled data, otherwise the comparisons were performed between the individual transgenic and control line. Both are reported. Also the results from 35S-PtCOMT lines (23 and 44) or UbB1-PtCOMT lines (65 and 130) were combined and pooled data was tested.

The RGR values obtained from the experiments were first scaled between 0 and 1 and then transformed with arcsin square transformation. Statistical analyses were performed with the analysis of covariance, and the possible impact of the varying initial weight of the larvae in RGR values was taken into account by using the initial larvae weight as a covariate. The Šidák's t test (that adjusts the significance level for multiple comparisons) was used for the pairwise comparisons of the estimated marginal means.

The leaf vein consumption data was transformed with arcsin square transformation and analysed either with analysis of variance or with non-parametric Kruskal-Wallis test. For the pairwise comparisons, the Dunnett's or Tukey's HSD test was used. The SPSS 11.0 statistical software was applied for all analyses.

Results

Molecular characterisation of transgenic birch lines

The *PtCOMT* gene (pRT99/35S-PtCOMT and pRT99/UbB1-PtCOMT constructs) was stably integrated into the genome of regenerated birch lines, and there were up to five copies of the *PtCOMT* gene in individual transgenic lines (Fig. 2a, b). The gene functioning was evidenced in leaves, phloem and xylem of all the transgenic lines. In all the sample types, the size of the UbB1-*PtCOMT* transcript was bigger than that of the 35S-*PtCOMT* transcript (Fig. 3).

Lignin analyses

Leaf lignin analyses indicated variation in S/G ratio between the lines (ANOVA; $F_{4,20} = 61.41$, $P < 0.001$) (Table 1), and pairwise comparisons showed significantly decreased S/G ratio in leaves of the 35S-PtCOMT lines when compared to the control (Tamhane's T2; $P < 0.001$ and $P = 0.003$ for the lines 23 and 44, respectively). In the UbB1–PtCOMT lines, no changes in the S/G ratio were observed. These observations were in line with the results on stem wood (Table 1).

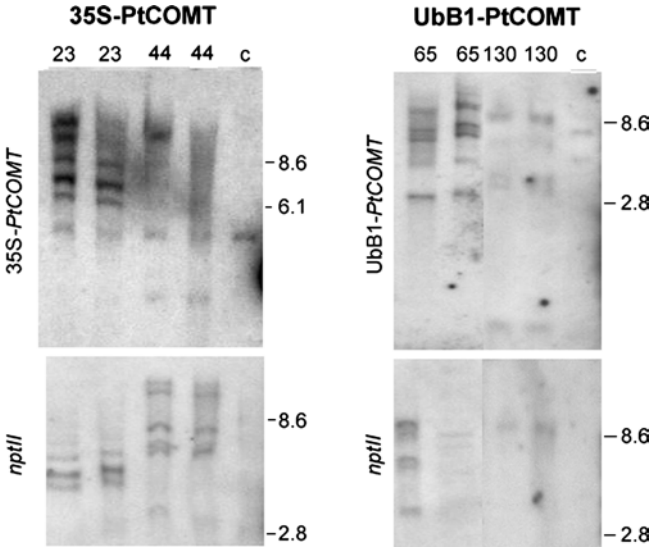

Fig. 2 a, b Verification of transgene integration in the 35S-PtCOMT lines (23 and 44) and UbB1-PtCOMT lines (65 and 130) by Southern blot analysis. Genomic DNA was digested with *Sac*I. Lanes marked with the line number, C = non-transgenic control, probes (35S-PtCOMT, UbB1-PtCOMT, nptII) shown on the left, molecular weight marker position as kilobase pairs shown on the right

The total and Klason lignin content in leaves varied between the lines (ANOVA; $F_{4,20} = 9.53$, $P < 0.001$ and $F_{4,20} = 14.79$, $P < 0.001$). The only observed difference was total and Klason lignin content in the line 130 being lower when compared to the other transgenic lines (Table 1), but not differing from the control. The nitrogen content of acid-insoluble Klason lignin in leaves of the lines 23, 44, 65 and control was at the same level, being 2.68, 2.76, 2.81 and 2.44% of air-dry weight, respectively. In the line 130, it was slightly higher, 3.47%.

Food selection

In food selection experiment of *A. punctulata* larvae (test of heterogeneity; $P > 0.05$), the data of individual transgenic lines indicated that the larvae preferred leaves of transgenic genotypes ($\chi^2 = 14.95$, df = 1, $P < 0.001$) (Fig. 4a). Individual comparisons, transgenic versus control line, indicated *A. punctulata* larvae to select leaves of the line 23 ($\chi^2 = 10.80$, df = 1, $P = 0.001$) and of the line 130 ($\chi^2 = 6.53$, df = 1, $P = 0.011$). Furthermore, the pooled data of the lines 23 and 44 revealed the preference for leaves of the 35S-PtCOMT-lines over control ($\chi^2 = 13.07$, df = 1, $P < 0.001$).

In *C. cinctaria* and *T. carpinata* experiments (for both test of heterogeneity; $P < 0.05$), individual comparisons indicated *C. cinctaria* larvae to prefer leaves of the line 130 over control ($\chi^2 = 8.53$, df = 1, $P = 0.004$) (Fig. 4b).

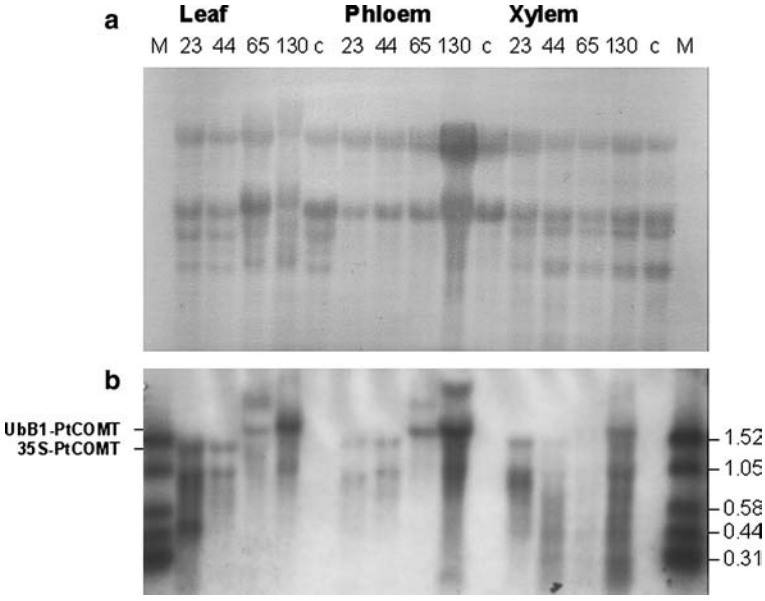

Fig. 3 Northern blot analysis indicating RNA loading (**a**, methyleneblue staining) and the *PtCOMT* gene expression (**b**) in leaves, phloem and developing xylem of the 35S-lines (23 and 44) and UbB1-lines (65 and 130). C = non-transgenic control, M = molecular weight marker, size shown as kilobase pairs on the right

T. carpinata larvae selected transgenic and control leaves equally (Fig. 4c). *A. alni* and *Phyllobius* spp. did not show any preference for leaves of different birch lines (Fig. 4d, e).

Relative growth rate (RGR)

The RGR of the tested herbivores on transgenic birch leaves did not differ compared to RGR on control leaves (Šidák's *t* test, $P \geq 0.136$ for *A. punctulata*, $P \geq 0.156$ for *T. carpinata* and $P \geq 0.746$ for *C. cinctaria*). In the cases of *A. punctulata* and *T. carpinata* larvae, the RGR values varied depending on the transgenic line (ANOVA; $F_{4,56} = 3.70$, $P = 0.010$ and $F_{3,48} = 3.07$, $P = 0.036$) but not in the case of *C. cinctaria* (Fig. 5c). *A. punctulata* larvae grew better when they fed on leaves of the line 130 than on the leaves of the lines 23 or 44 (Šidák's *t* test, $P = 0.007$ and $P = 0.015$) (Fig. 5a). The highest RGR of *T. carpinata* was measured on leaves of the line 65 and the lowest on leaves of the line 23, although this difference was not statistically significant (Šidák's *t* test, $P = 0.070$) (Fig. 5b).

The leaf vein consumption

The leaf vein consumption was determined on both food selection and RGR experiments. In general, geometrid larvae utilised more leaf veins than coleopteran species (Fig. 6a, b). Based on the food selection data, the feeding behaviour of lepidopterans was not significantly altered due to the lignin modification, as presented in Table 2. In the case of the coleopterans, *Phyllobius* spp. consumed significantly more leaf veins of the line 23 than the veins of the other lines (Table 2). There were no significant differences in leaf vein consumption based on the RGR data (Table 2).

Table 1 Quantitative (%/DW) and qualitative (S/G ratio) leaf and stem wood lignin analyses of PtCOMT birch lines and control. Lines 23 and 44 are under 35S-CaMV promoter and lines 65 and 130 under UbB1-promoter. Tests of quantitative data are based on arcsin transformed data.

Line	Klason lignin (%) mean ± SE		Acid-soluble lignin (%) mean ± SE	Total lignin (%) mean ± SE	Thioacidolysis: S/G mean ± SE	
	Leaves	2-year-old stem[e]	Leaves	Leaves	Leaves	2-year-old stem[f]
23	17.33 ± 0.67^{a}	23.48 ± 0.35^{a}	3.62 ± 0.10^{a}	20.95 ± 0.74^{a}	0.26 ± 0.06^{a}	0.63 ± 0.02
44	15.63 ± 0.37^{a}	23.28 ± 0.48^{a}	$3.02 \pm 0.15^{b,d}$	$18.65 \pm 0.37^{a,b}$	0.66 ± 0.05^{b}	0.71 ± 0.03
65	16.45 ± 0.17^{a}	26.07 ± 0.26^{b}	2.72 ± 0.07^{b}	19.17 ± 0.22^{a}	1.05 ± 0.04^{c}	2.14 ± 0.02
130	12.87 ± 0.20^{b}	–	4.14 ± 0.04^{c}	17.01 ± 0.23^{b}	1.03 ± 0.02^{c}	–
Control	$15.01 \pm 0.56^{a,b}$	$24.65 \pm 0.29^{a,b}$	3.15 ± 0.06^{d}	$18.16 \pm 0.56^{a,b}$	1.05 ± 0.04^{c}	2.27 ± 0.02

–The lignin data are not available from the same developmental stage of the line 130 compared to the other lines

[a,b,c,d]The significantly different means ($P < 0.05$) are marked with differing letters

[e]The stem wood lignin analyses are described in Aronen et al. (2003)

[f]The stem wood lignin analyses are described in Aronen et al. (2003). The S/G results are based on two measurements, and statistical analysis were not performed

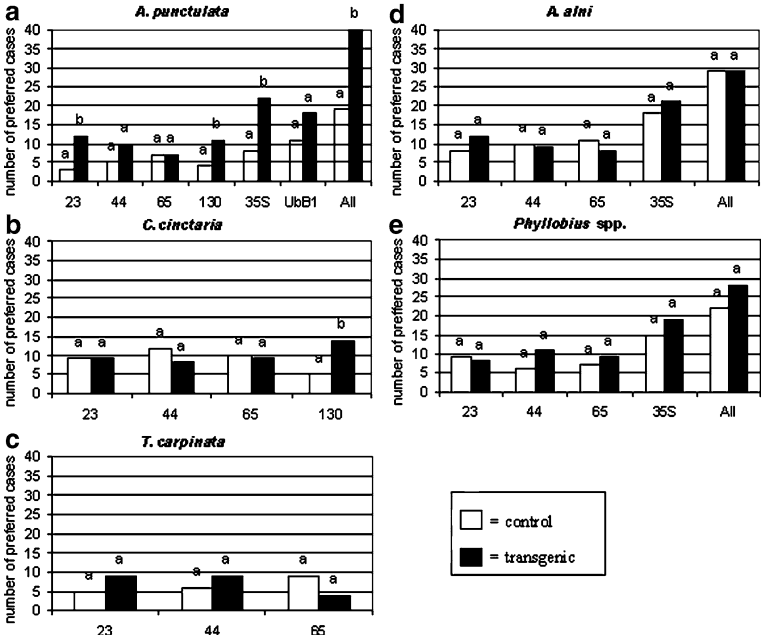

Fig. 4 **a–e** Food selection results on leaves of the lines 23 and 44 derived from the 35S-PtCOMT transformation and of the lines 65 and 130 derived from the UbB1-PtCOMT transformation. UbB1 = pooled data from the lines 65 and 130. 35S = pooled data from the lines 23 and 44. All = pooled data from all the tested lines. When supported with the test of heterogeneity, the results obtained from individual lines are presented as a pooled data. Significant differences (χ^2 -test, $P < 0.05$) are indicated with differing letters

Discussion

We found a decreased ratio of S/G lignin monomers in leaf lignin of the 35S-PtCOMT (23 and 44) but not of the UbB1-PtCOMT (65 and 130) silver birch lines. These results are in accordance with our results on stem wood lignin (Aronen et al. 2003). 35S-CaMV derived from plant virus and the intron-containing UbB1 derived from sunflower are both constitutive promoters. As found in mRNA analyses and discussed in Aronen et al. (2003), the *PtCOMT* transcript size in the present transgenic birch lines seems to be different under the control of the two promoters. Although not verified with enzyme activity comparisons, the higher molecular weight of the UbB1-transcripts compared to 35S-transcripts illustrates the possible difference in post-transcriptional events, which might further contribute to varying effects in lignin biosynthesis and thus different lignin composition in these lines. In the 35S-PtCOMT lines, the S-lignin synthesis was silenced in co-ordinate ways in leaves and in stem xylem apparently due to the co-suppression based on the sequence homology between the *Populus* and birch *COMT* genes.

The binding characteristics of S- and G-monomers are different. The interunit linkages, β-5 and 5–5, make G-lignin more resistant to delignification in pulping process, compared to S-lignin characterised by a higher proportion of β-O-4 linkages (Baucher et al. 1998). The S/G structure can affect digestibility of crop plants, although the results are controversial. According to Jung et al. (1999), the lack of S-lignin deposition in ferulate-5-hydroxylase-deficient *Arabidopsis* mutant did not alter the cell wall degradability. The COMT-suppressed tobacco (Vailhé et al. 1996), tropical legume (Rae et al. 2001) or COMT-deficient *Arabidopsis* mutant (Goujon

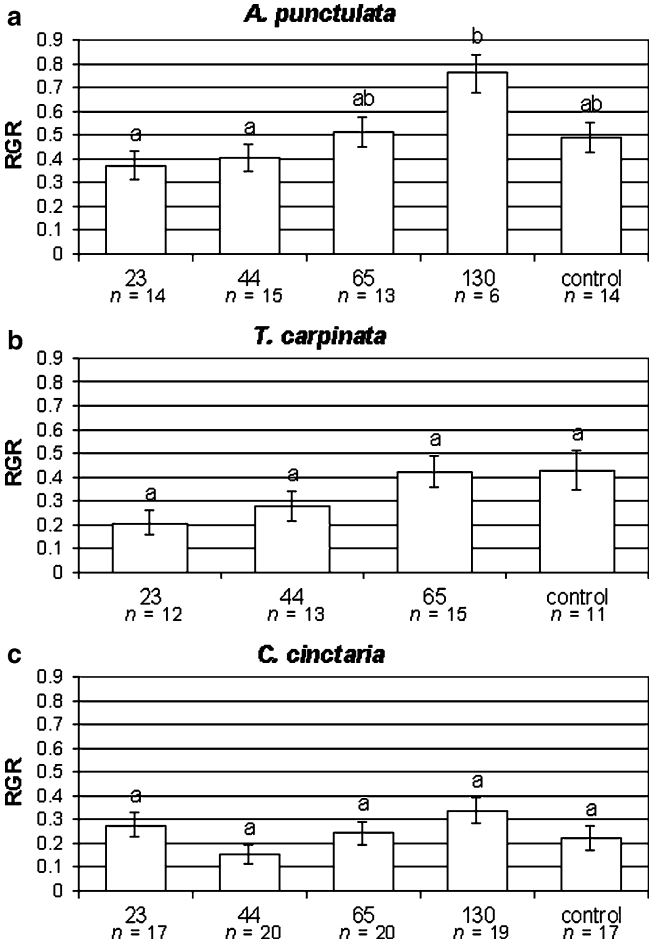

Fig. 5 **a–c** Covariate adjusted relative growth rates (RGR, larval weight as a covariate) of geometrid larvae of *A. punctulata*, *T. carpinata* and *C. cinctaria* (estimated marginal mean ± SE). Significant differences among means ($P < 0.05$) are marked with differing letters (Šidák's *t* test)

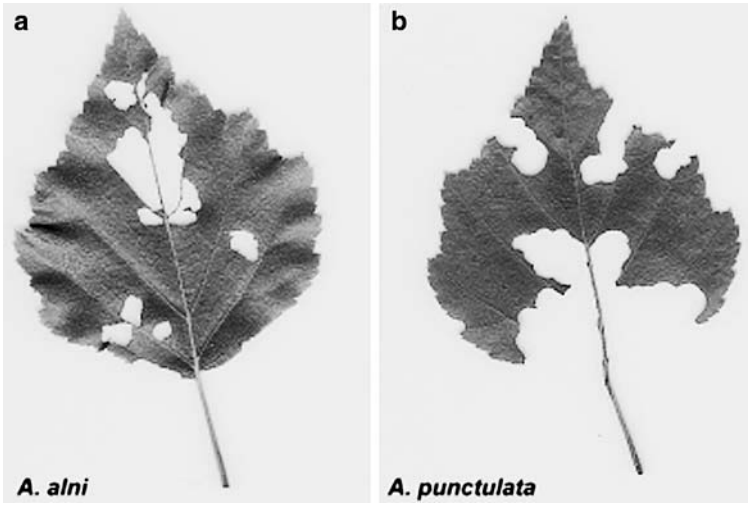

Fig. 6 **a**, **b** Feeding patterns of *Agelastica alni* (leaf beetle) and *Aethalura punctulata* (geometrid moth larvae). Leaf beetles consumed mainly leaf tissues between veins, whereas the geometrid larvae were able to feed on the vascular tissues as well

et al. 2003) showed that the reduction in S-lignin is associated with the improved plant digestibility. On the other hand, as reported by Guo et al. (2001), the improved digestibility of transgenic alfalfa lines was suggested to result from a combination of the reduced lignin level and an increased S/G ratio. In woody plants, there are no existing studies concerning the possible impact of COMT-modification on leaf digestibility.

In this study, growth performance of lepidopteran larvae did not differ significantly between control and transgenic birch lines. The lowest growth rates of *A. punctulata* and *T. carpinata* were measured when larvae were fed on leaves with increased G-lignin (lines 23 and 44). This might mean that the increased G-lignin could at certain level impair the digestibility of leaves for insect herbivores, possibly due to the limitations in cell wall degradation and further carbohydrate and/or protein utilisation (Jung and Deetz 1993; Baucher et al. 1998; Lam et al. 2003). Most of the leaf lignin is deposited in veins (Stafford 1988). In herbivores such as lepidopteran larvae in the present study able to consume leaf veins in their diet (Saalas 1949), the lignin modification seemed not to alter the leaf vein-consumption compared to control and thus the modified lignin supplied with the diet resulted on average, but not significantly, the lowest growth rates of larvae. It can, however, not be excluded that the found variation in RGR values was within the natural variation.

We found that *A. punctulata* larvae preferred leaves of the transgenic lines 130 and 23. The preference for leaves of the line 130 may be explained by different plant quality due to the belated growth rhythm resulting in relatively younger leaf material in this line. Corresponding preference was also found in the *C. cinctaria* experiment. The preference for leaves of the line 23 by *A. punctulata* larvae is probably also not associated with lignin modification, because there was no evidence for the preference of the other 35S-PtCOMT line (44) with comparable S/G lignin modifications. In general, several leaf factors such as water content, nutritious compounds (sugars and proteins) and secondary metabolites e.g., condensed tannins in birch species (Ayres et al. 1997; Mutikainen et al. 2000) and their potential interactions affect herbivore feeding (Ossipov et al. 2001; Haukioja 2003), and we recognise that these factors may contribute to our results.

The Klason lignin content (around 16% per dry weight) of leaves of the lines 23, 44 and 65 did not differ from control. Previous report indicates similar total lignin levels in birch leaves (Voipio and Laakso 1992) as

Table 2 The leaf vein consumption measured as a mean percentage of consumed leaf veins (%) ± SE on food selection and RGR experiments of insect herbivores

Line	Food selection					RGR		
	A. punctulata n = 112	*C. cinctaria* n = 140	*T. carpinata* n = 65	*A. alni* n = 111	*Phyllobius spp* n = 78	*A. punctulata* n = 64	*C. cinctaria* n = 98	*T. carpinata* n = 54
23	34.8 ± 5.19^{a}	60.7 ± 5.48^{a}	30.2 ± 5.03^{b}	9.5 ± 1.95^{d}	16.3 ± 3.09^{e}	57.7 ± 6.34^{a}	60.3 ± 7.40^{a}	24.1 ± 3.29^{d}
44	37.7 ± 6.14^{a}	49.7 ± 6.43^{a}	$12.0 \pm 4.13^{b,c}$	8.5 ± 2.08^{d}	6.5 ± 2.11^{f}	59.2 ± 4.22^{a}	51.8 ± 6.57^{a}	25.2 ± 4.38^{d}
65	36.7 ± 7.01^{a}	44.5 ± 6.84^{a}	12.3 ± 4.71^{c}	7.7 ± 2.04^{d}	7.3 ± 2.85^{f}	66.2 ± 5.06^{a}	57.9 ± 7.01^{a}	35.8 ± 5.98^{d}
130	42.9 ± 5.15^{a}	61.5 ± 7.77^{a}	NT	NT	NT	55.7 ± 6.49^{a}	46.5 ± 7.58^{a}	NT
Control	29.8 ± 2.71^{a}	47.9 ± 3.87^{a}	$16.7 \pm 2.87^{b,c}$	8.2 ± 1.11^{d}	8.1 ± 1.46^{f}	56.9 ± 5.08^{a}	53.0 ± 6.86^{a}	35.5 ± 4.23^{d}

[a]No significant differences among lines by ANOVA
[b,c]Significant differences among lines marked with differing letters by Tukey's test ($P = 0.029$)
[d]No significant differences among lines by Kruskal-Wallis test
[e,f]Significant difference among lines marked with differing letter by Kruskal-Wallis test ($P = 0.013$)
NT not tested

in the present study. The leaves of the line 130 contained less Klason lignin and more acid soluble lignin than controls, although not significantly. This may be related to the belated growth habit of the line and still ongoing lignification at the sampling date. The best growth performances of lepidopteran larvae of *A. punctulata* and *C. cinctaria* on leaves of this line may support the view that the decreased lignin content is generally associated with the improved forage digestibility (Sewalt et al. 1997; Guo et al. 2001; He et al. 2003).

The use of transgenic trees has evoked the concern about the unintended side effects or pleiotrophic effects of transgenes. In the present study, the differing growth performance and preference results between transgenic lines might be explained by the site effect of transgene (e.g., the differing results of the two 35S-PtCOMT lines 23 and 44) or differing growth characteristics of transgenic line (130).

As a conclusion, the sense-suppression of *COMT* in silver birch (35S-PtCOMT lines) was found to alter the S/G composition of lignin in leaves, and these alterations were in line with the changes in xylem. Although the feeding preferences of insect herbivores varied among the birch lines, feeding preferences seemed not to be associated with lignin modification. The growth performances of geometrid larvae did not differ from the control leaves. Controlled feeding experiments like this can thus provide the opportunity to study specific ecological interactions between lignin-modified tree species and insect herbivores. For a more comprehensive understanding of these interactions, evaluations out in the field are needed.

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