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NOTE

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Profiling of phenylpropanoid monomers in developing xylem tissue of transgenic aspen (*Populus tremuloides*)

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Abstract Here we describe alterations in the cinnamate/monolignol pathway in three transgenic aspen lines: one with downregulated expression of 4-coumarate:CoA ligase (4CL), one with upregulated expression of coniferaldehyde 5-hydroxylase (CAld5H), and a 4CL downregulated/CAld5H upregulated line. Compared with the wild type, the 4CL downregulated line showed significantly increased levels of *p*-hydroxycinnamic acids such as *p*-coumaric, ferulic, and sinapic acids. In contrast, the CAld5H upregulated line had increased content of *p*-coumaryl and 5-hydroxyconiferyl alcohols. In the 4CL downregulated line, it was likely that most hydroxycinnamic acids were glycosylated. These results strongly suggest that the downregulation of 4CL and upregulation of CAld5H disrupt the metabolic flow through the cinnamate/monolignol pathway and thus alter the amount and structure of its final product, lignin.

Key words Aspen · Transgenic · 4-Coumarate:CoA ligase · Coniferaldehyde 5-hydroxylase · Metabolic profiling

Introduction

Lignin is one of the major polymers in the secondary xylem of trees. It provides the rigidity and structural support that enable water transport in the vascular system. Regardless of its importance during growth, lignin becomes problematic in postharvest cellulose-based wood processing because it must be removed from cellulose at great expense.[1]

In angiosperm trees, lignin is composed of a mixture of syringyl and guaiacyl moieties. These units are generated by the oxidative coupling of sinapyl and coniferyl alcohols. Because the guaiacyl moiety promotes the cross-linkages that increase lignin's resistance to degradation, syringyl-rich lignin is more degradable than guaiacyl-rich lignin. As a result, angiosperm lignin can be degraded with considerably less energy and lower chemical costs than gymnosperm lignin. Therefore, genetically engineered trees with a high syringyl/guaiacyl (S/G) ratio and a low lignin content would facilitate wood processing for pulping.[1]

The immediate precursors of lignin, i.e., monolignols, are biosynthesized via the cinnamate/monolignol pathway (Fig. 1). Downregulation of several genes in the pathway has resulted in reduced lignin production, but such modifications often cause pleiotropic effects in engineered plants.[2] In contrast, Hu et al. demonstrated that transgenic aspen with downregulated 4-coumarate:CoA ligase (4CL) accumulated less lignin and grew better than the control.[2] Later, genetically engineered aspens with a high syringyl/guaiacyl (S/G) ratio and a reduced lignin content were produced by 4CL downregulation and coniferaldehyde 5-hydroxylase (CAld5H) upregulation.[1]

The 4CL downregulated aspen with less lignin contained more cellulose than control aspen.[1,2] In contrast, the xylem in the CAld5H upregulated aspen matured more rapidly than that of the control.[1] These data suggested that genetic modification of a metabolic pathway changes not only the target pathway but other metabolic pathways as well.

Metabolic profiling, in which metabolites are measured and compared, is a valuable technique for studying changes in metabolic pathways. Previously, Sakakibara et al. used a stable isotope dilution method and gas chromatography-mass spectrometry (GC-MS) to carry out metabolic profiling of the cinnamate/monolignol pathway in developing seeds of *Carthamus tinctorius*, which accumulates significant amounts of lignin.[3] This system may be the first step to

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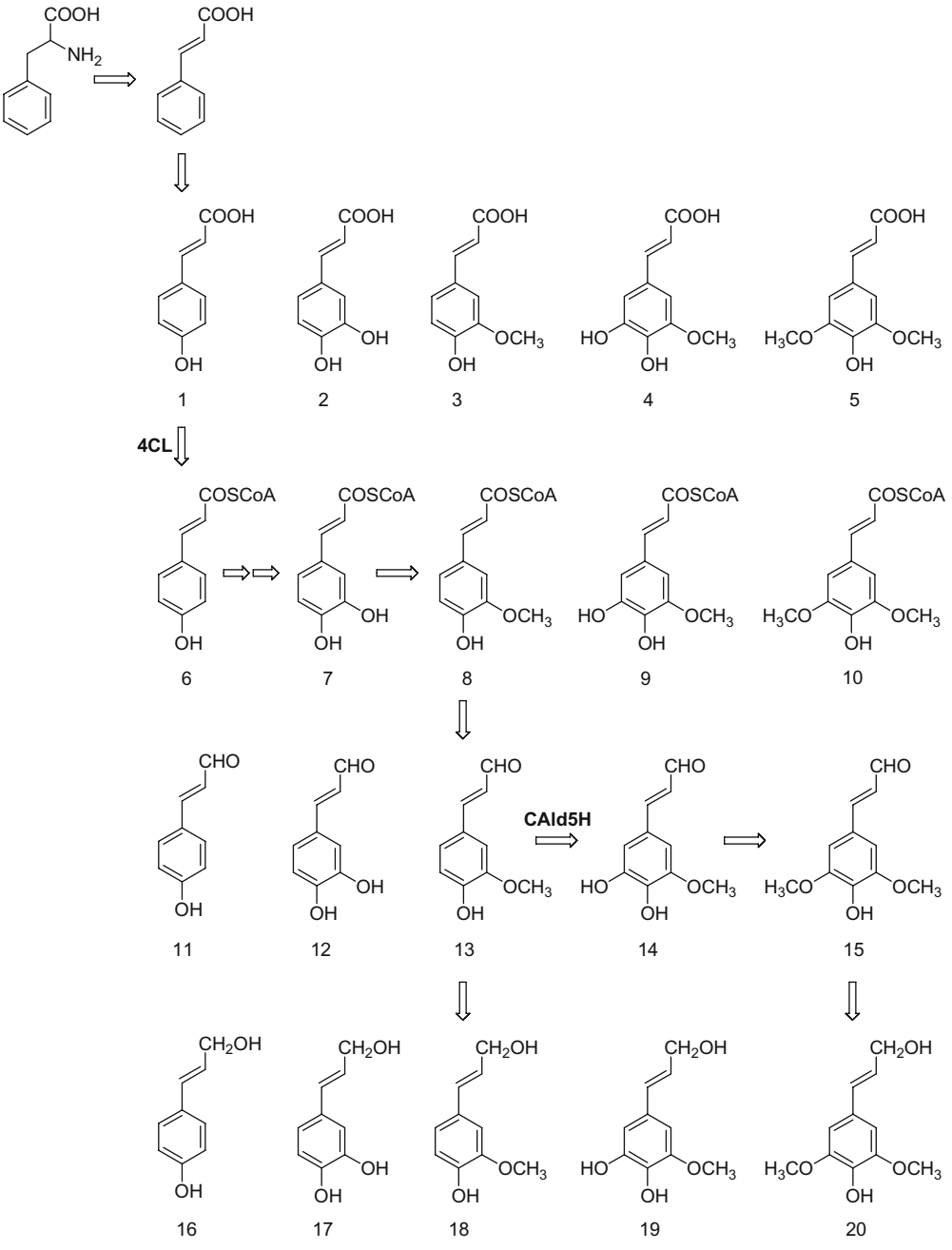

Fig. 1. The cinnamate/monolignol pathway and }its possible intermediates: *1*, *p*-coumaric acid; *2*, caffeic acid; *3*, ferulic acid; *4*, 5-hydroxyferulic acid; *5*, sinapic acid; *6*, *p*-coumaroyl-CoA; *7*, caffeoyl-CoA; *8*, feruloyl-CoA; *9*, 5-hydroxyferuloyl-CoA; *10*, sinapoyl-CoA; *11*, coumaraldehyde; *12*, caffealdehyde; *13*, coniferaldehyde; *14*, 5-hydroxyconiferaldehyde; *15*, sinapaldehyde; *16*, *p*-coumaryl alcohol; *17*, caffeyl alcohol; *18*, coniferyl alcohol; *19*, 5-hydroxyconiferyl alcohol; *20*, sinapyl alcohol

characterize xylem development at the metabolic level. Using this method, we may be able to determine the mechanisms that underlie cellulose accumulation and the accelerated maturation of xylem observed in 4CL downregulated and CAld5H upregulated aspen.

Here, we describe the metabolic profiles of three transgenic aspens displaying altered lignin deposition. Using the stable isotope dilution method, we analyzed aspens with downregulated 4CL expression and upregulated CAld5H expression. Compared with the wild type, the 4CL downregulated line had increased *p*-hydroxycinnamic acids such as *p*-coumaric, ferulic, and sinapic acids. In contrast, the CAld5H upregulated line had increased content of *p*-coumaryl and 5-hydroxyconiferyl alcohols. In the 4CL downregulated line, β-glucosidase treatment increased the amount of *p*-hydroxycinnamic acids. Together with our previous reports, our results indicate that altering expression of 4CL and CAld5H changes the metabolic flow of the cinnamate/monolignol pathway and ultimately affects the amount and structure of the final product of the pathway, lignin.

Materials and methods

Plant materials

We used three transgenic aspen (*Populus tremuloides*) lines: line 23 (4CL downregulated), line 93 (CAld5H upregulated), and line 141 (4CL downregulated/CAld5H upregu-

lated).[1] The three lines were grown in a greenhouse at the Forest Biotechnology Group, North Carolina State University (Raleigh, NC, USA) and were maintained for 2 years. We collected developing xylem tissue by scratching with a sharp blade and immediately immersed the sample in liquid nitrogen. The sample was stored in liquid nitrogen until use.

Extraction of metabolites

The developing xylem was ground in liquid nitrogen with a mortar and pestle. The powdered xylem (100 mg) was weighed immediately and transferred into a prechilled 2-ml microcentrifuge tube. Methanol (1.5 ml) and the synthetic deuterium-labeled phenylpropanoid monomers (4 µl of 1 mg/ml methanol solution) were added. The synthesis of the added monomers, *p*-[3,5,7-$^2H_3$]coumaric acid, [2,5,7-$^2H_3$]-caffeic acid, [2,5-$^2H_2$, 3-OC^2H_3]ferulic acid, [3-OC^2H_3]sinapic acid, *p*-[3,5,7-$^2H_2$]coumaraldehyde, [3-OC^2H_3]coniferaldehyde, [3-OC^2H_3]-5-hydroxyconiferaldehyde, [3-OC^2H_3,]sinapaldehyde, *p*-[3,5,7-$^2H_3$]coumaryl alcohol, [3-OC^2H_3]coniferyl alcohol, [3-OC^2H_3]-5-hydroxyconiferyl alcohol, and [3-OC^2H_3]sinapyl alcohol were described previously.[3]

The mixture was vigorously vortexed for 3 min, heated at 60°C for 10 min, and then centrifuged at 10 000 *g* for 10 min at room temperature. An aliquot of the supernatant (1 ml) was filtered through a 0.45-µm Millex-HN syringe filter (Millipore) and evaporated. The resulting residue was suspended in 0.1% acetic acid (0.5 ml) and extracted with ethyl acetate (0.5 ml). An aliquot (0.1 ml) of the organic layer was transferred to a new glass vial and evaporated.

Metabolite analysis

N,*O*-Bis(trimethylsilyl)acetamide (BSA, 10 µl) was added to the ethyl acetate extract, and the mixture was heated at 60°C for 10 min for derivatization. The mixture was analyzed on a ThermoFinnigan Trace GC equipped with a Polaris Q ion-trap mass spectrometer (GC-MS). An HP-1 column (30 m × 0.32 mm × 0.25 µm; Agilent Technologies) was connected to the gas chromatograph. The column was first set at 70°C for 5 min, and then the temperature was increased at 5°C/min up to 310°C. The carrier gas (He) was eluted at a flow rate of 1 ml/min.

β-Glucosidase treatment

The methanol extraction was carried out as already described in "Extraction of metabolites." Methanol was evaporated off, and β-glucosidase from almonds (10 units, Sigma) in 0.5 ml 0.1 M sodium acetate buffer (pH 5.2) was added to the residue. The mixture was incubated at 37°C for 24 h and extracted with 0.5 ml ethyl acetate. The extract was evaporated off and derivatized with BSA as described above.

GC-MS data analysis

The chromatograms of molecular ions were plotted using the Xcalibur software included with the GC-MS system. We calculated the amount of endogenous unlabeled metabolites based on the peak areas of molecular ions of unlabeled and labeled compounds, as described previously.[4]

Results

Phenylpropanoid monomers in wild-type aspen

Using GC-MS, we analyzed ethyl acetate extract prepared from a methanolic extract of developing xylem of wild-type aspen. We found several phenylpropanoid monomers, which were identified as trimethylsilyl ethers by comparison of their mass spectra and retention times on the GC with those of authentic samples [*p*-coumaric acid (TMS ether): MS *m/z* (EI) 308 (M^+, 37.2%), 293 (100), 249 (62.6), 219 (31.9), 179 (16.6), t_R = 30.6 min; caffeic acid (TMS ether): MS *m/z* (EI) 396 (M^+, 26.8%), 381 (13.8), 307 (3.0), 219 (100), 191 (8.3), t_R = 34.6 min; ferulic acid (TMS ether): MS *m/z* (EI) 338 (M^+, 83.6%), 323 (100), 308 (78.3), 293 (79.3), 249 (51.7), t_R = 33.5 min; sinapic acid (TMS ether): MS *m/z* (EI) 368 (M^+, 64.1%), 353 (55.9), 338 (100), 323 (55.8), 279 (27.8), t_R = 36.1 min; coniferyl alcohol (TMS ether): MS *m/z* (EI) 324 (M^+, 100%), 309 (30.6), 293 (85.6), 235 (22.4), 204 (82.8), t_R = 30.7 min; sinapyl alcohol (TMS ether): MS *m/z* (EI) 354 (M^+, 100%), 324 (61.0), 293 (22.6), 264 (21.4), 234 (79.1), t_R = 33.4 min].

Under these conditions, we did not detect other possible intermediates in the cinnamate/monolignol pathway, including 5-hydroxyferulic acid, *p*-coumaraldehyde, caffealdehyde, coniferaldehyde, 5-hydroxyconiferaldehyde, sinapaldehyde, *p*-coumaryl alcohol, caffeyl alcohol, and 5-hydroxyconiferyl alcohol. We did not analyze CoA thioesters (*p*-coumaroyl-, caffeoyl-, feruloyl-, 5-hydroxyferuloyl-, and sinapoyl-CoAs) because GC-MS is not suitable for determination of these compounds.[3]

Determination of *p*-hydroxycinnamic acids, *p*-hydroxycinnamaldehydes, and *p*-hydroxycinnamyl alcohols in wild-type and transgenic aspens

Using a stable isotope dilution method,[3] we quantified 12 phenylpropanoid monomers including *p*-hydroxycinnamic acids, *p*-hydroxycinnamaldehydes, and *p*-hydroxycinnamyl alcohols in developing xylem tissue of transgenic (lines 23, 93, and 141) and wild-type lines (Fig. 2). Compared with the wild type, line 23 had increased amounts of *p*-hydroxycinnamic acids but decreased amounts of sinapaldehyde and sinapyl alcohol. In line 96, there was a marked increase in caffeic acid, and *p*-coumaric and ferulic acids were also increased. Notably, line 96 had lower levels of sinapaldehyde but higher levels of sinapyl and coniferyl alcohols. In line 141, there were increased levels of *p*-coumaric and caffeic acids, unchanged ferulic and sinapic acids contents,

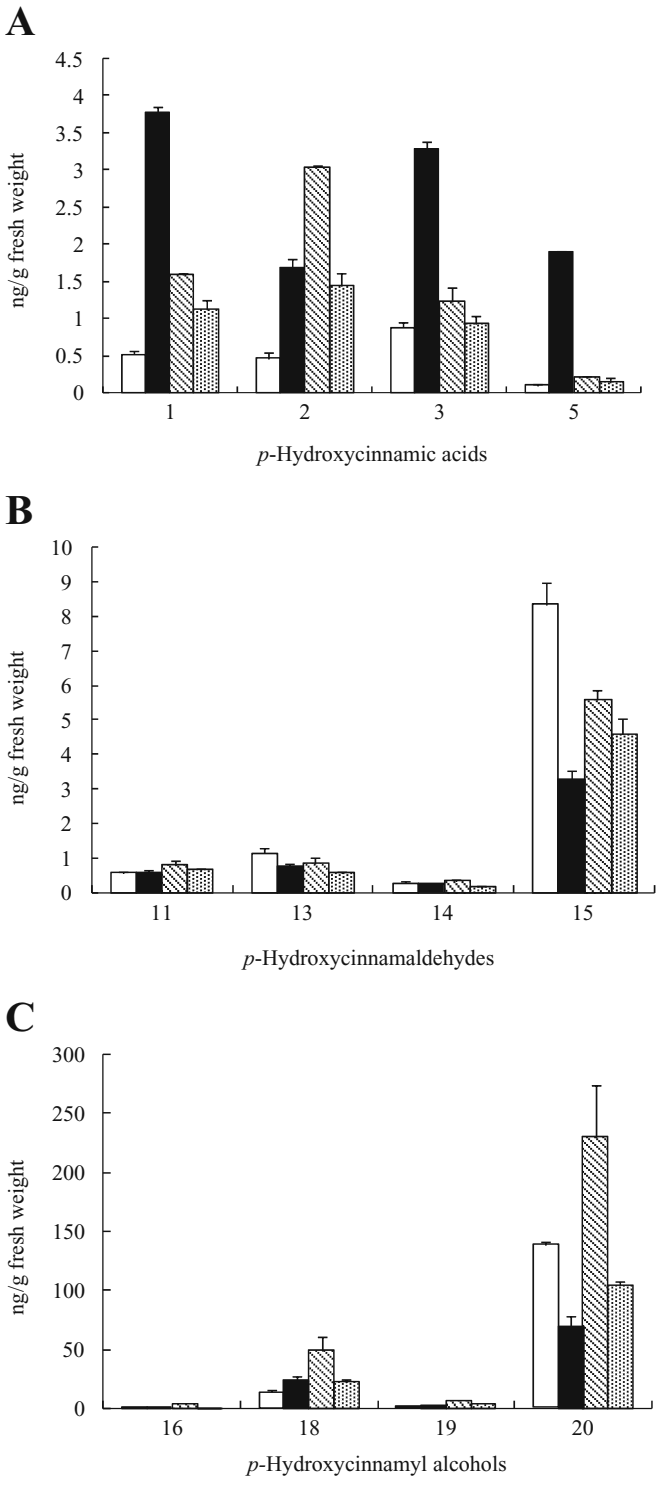

Fig. 2. Phenylpropanoid monomers in xylem of wild-type and transgenic aspens. **A** *p*-hydroxycinnamic acids; **B** *p*-hydroxycinnamaldehydes; **C** *p*-hydroxycinnamyl alcohols. *Open bar*, wild type; *solid bar*, line 23; *hatched bar*, line 93; *shaded bar*, line 141. Note that numbers on *x*-axis represent compounds in Fig. 1

and decreased sinapyl alcohol and sinapaldehyde contents.

Increased yield of *p*-hydroxycinnamic acids after β-glucosidase treatment of 4CL downregulated line

We treated methanolic extract of developing xylem tissue from line 23 (the 4CL downregulated line) and the wild-type with β-glucosidase and analyzed the phenylpropanoid monomers that were produced in each reaction. After β-glucosidase treatment of line 23, there were significant increases in ferulic and sinapic acids, and slight increases in *p*-coumaraldehyde, coniferaldehyde, and sinapaldehyde (Fig. 3). Coniferyl and sinapyl alcohol levels largely decreased.

Discussion

Here, we report metabolic profiles of several transgenic aspens. To construct metabolic profiles of the developing xylems, we used a stable isotope dilution method, with corresponding deuterium-labeled compounds as internal standards, and precisely measured the metabolites in the cinnamate/monolignol pathway. The advantages of this technique are that it is highly sensitive, and inclusion of labeled standards can quantify the loss of target compounds during extraction and derivatization.[3] For example, when internal standards labeled with deuterium were not added to the methanol extraction procedure, sinapaldehyde was not detected in wild-type aspen xylem (data not shown), but sinapaldehyde was easily quantified when internal standards were added before methanol extraction (see Fig. 2). Because sinapaldehyde is an unstable α,β-unsaturated phenolic aldehyde,[5] endogenous sinapaldehyde in developing xylem tissue might be degraded during extraction and/or derivatization. This result, in addition to our previous result,[3] shows that the stable isotope dilution method accurately quantifies unstable metabolites.

Using the stable isotope dilution method, we quantified the four *p*-hydroxycinnamic acids, four *p*-hydroxycinnamaldehydes, and four *p*-hydroxycinnamyl alcohols in developing xylem tissue of control and transgenic aspen lines. Previously, Li et al. reported lignin contents of 22.2% ± 0.8% in the control line, 14.4% ± 0.5% in the 4CL downregulated line 23, 21.1% ± 0.4% in the CAld5H upregulated line 93, and 10.7% ± 0.4% in the 4CL downregulated/CAld5H upregulated line 141.[1] The lignin S/G ratios of these four lines were 2.2, 2.2, 5.5, and 2.7, respectively.[1] In addition, xylem 4CL activity with caffeic acid as substrate was reduced 88% in line 23 and 91% in line 141, and xylem CAld5H activity with coniferaldehyde as substrate was increased 2.8 fold in line 93 and 1.8 fold in line 141.[1] These results suggest that 4CL downregulation and/or CAld5H upregulation disrupt the normal metabolic flow of the cinnamate/monolignol pathway. As a result, there are changes in the amounts and ratios of monolignols, which are the immediate precursors of lignin. Together, these alterations

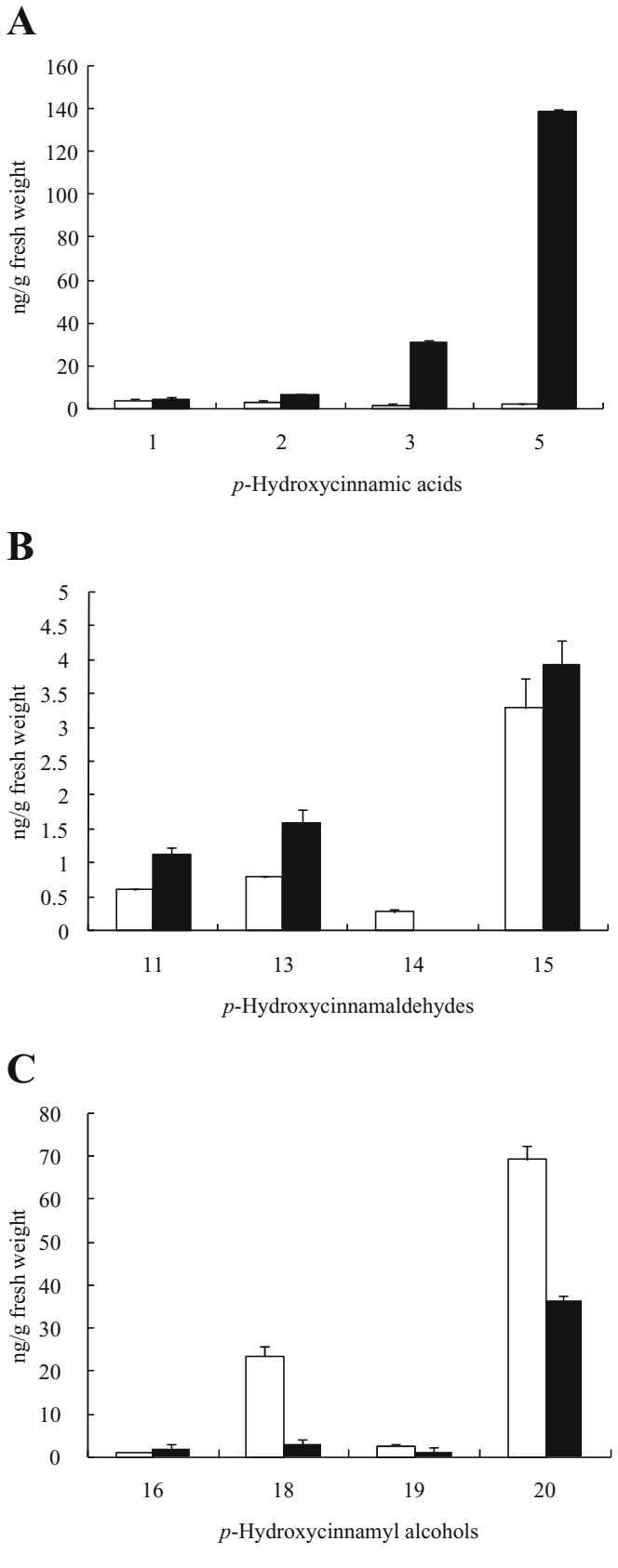

Fig. 3. Yield of phenylpropanoid monomers after β-glucosidase treatment of methanol extract from xylem of wild-type and line 23 aspens: **A** *p*-hydroxycinnamic acid; **B** *p*-hydroxycinnamaldehydes; **C** *p*-hydroxycinnamyl alcohols. *Open bar*, wild-type; *solid bar*, line 23. Note that numbers on *x*-axis represent compounds in Fig. 1

in the biosynthetic pathway result in structural and quantitative changes to the end product, lignin.

Our results are consistent with this speculation. Compared with the wild type, the three transgenic lines (23, 93, and 141) showed different profiles of phenylpropanoid monomer accumulation. Line 23, the 4CL downregulated line, showed increased accumulation of all *p*-hydroxycinnamic acids measured (Fig. 2A). Treatment of methanolic extract from the developing xylem of line 23 with β-glucosidase increased the yield of *p*-hydroxycinnamic acids, particularly ferulic and sinapic acids (Fig. 3A). Our data suggest that 4CL downregulation decreased the flux of *p*-coumaric acid to lignin and, alternatively, activated a shunt from *p*-coumaric to sinapic acids via caffeic, ferulic, and 5-hydroxyferulic acids, and probably the subsequent glycosylation. Similar observations have been reported previously in lignin-deficient caffeoyl-CoA *O*-methyltransferase-downregulated poplar, where the glucosides of caffeic and sinapic acids were significantly increased compared to the control.[6] The *p*-hydroxycinnamic acid glucosides might be formed as storage or detoxification products.[6]

In the 4CL downregulated line, there were reduced levels of coniferaldehyde, sinapaldehyde, and sinapyl alcohol but a slight increase in coniferyl alcohol. Other aldehydes and alcohols tested were not significantly different between the wild type and the 4CL downregulated line (see Fig. 2). In the proposed pathway for syringyl lignin biosynthesis,[7] the metabolic flux passes through coniferaldehyde, sinapaldehyde, and sinapyl alcohol, which are located downstream from *p*-coumaroyl-CoA metabolized by 4CL from *p*-coumaric acid. Therefore, 4CL downregulation may shut down the metabolic flux from *p*-coumaric acid to the corresponding CoA and, as a result, reduce the endogenous levels of coniferaldehyde, sinapaldehyde, and sinapyl alcohol. This action did not appear to affect other aldehydes and alcohols, except for coniferyl alcohol. The S/G ratio was unaltered in the 4CL downregulated line,[1] in spite of the decreased sinapyl alcohol and increased coniferyl alcohol content (Fig. 2).

The CAld5H upregulated line, line 93, showed reduced level of coniferaldehyde (Fig. 2). As CAld5H is responsible for the conversion of coniferaldehyde to 5-hydroxyconiferaldehyde, we can speculate that CAld5H upregulation reduces coniferaldehyde levels and increases the amount of the product, 5-hydroxyconiferaldehyde. However, endogenous levels of 5-hydroxyconiferaldehyde are very low, and are similar among the different lines; this may be because CAld5H has a turnover rate that is 6- to 50 times slower that those of the other two syringyl pathway enzymes,[8–10] and the current CAld5H upregulation cannot compensate for the slow turnover. Indeed, the xylem CAld5H activity (with coniferaldehyde as substrate) is increased less than 3 times in the CAld5H-overexpressing line 93.[1]

In line 93, the endogenous level of sinapyl alcohol was greatly increased, which was consistent with the high S/G ratio in this line. In addition, *p*-hydroxycinnamic acids and coniferyl alcohol were increased and sinapaldehyde was reduced. The reasons for this are unclear. Previously, we reported that line 93 showed accelerated maturation (wall

thickening and lignification) of xylem cells[1]; this may have a secondary impact in elevating endogenous levels of *p*-hydroxycinnamic acids and coniferyl alcohol.

Line 141 has downregulated expression of 4CL and upregulated expression of CAld5H. Metabolic profiling showed no significant change in the amounts of sinapic and ferulic acids compared to the wild type. It appears that the accumulation of sinapic and ferulic acids in 4CL downregulated lines is eliminated by the CAld5H upregulation in line 141. We can speculate that upregulation of CAld5H increases the activity of the metabolic pathway from coniferaldehyde to 5-hydroxyconiferadehyde, thus impacting on the large pool size of ferulic and sinapic acids produced by 4CL downregulation.

In conclusion, our present study showed that the metabolism of 4CL downregulated, CAld5H upregulated, and 4CL downregulated/CAld5H upregulated transgenic aspen lines differs from that of the wild-type. Genetic transformation altered the amount of metabolites in the cinnamate/monolignol pathway, resulting in changes in the amount and structure of its final product, lignin.

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