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Treatment of poplar callus with ferulic and sinapic acids II: effects on related monolignol biosynthetic enzyme activities

Abstract In a previous study we reported that administered ferulic acid (FA) was incorporated into guaiacyl and syringyl lignin in poplar callus (*Populus alba* L.) but administered sinapic acid (SA) was not. It is possible that SA may induce or enhance the biosynthesis of syringyl lignin. In this study, enzyme activities in lignin biosynthesis were assayed in FA- or SA-treated callus. The activity of 4-coumarate:coenzyme A ligase (4CL) toward FA in FA-treated callus was not affected, and its activity toward caffeic and sinapic acids was quite low. In addition, phenylalanine ammonia-lyase (PAL) activity, cinnamate:4-hydroxylase (C4H) transcription in the upstream steps of lignin biosynthesis, also was not affected. 4CL from SA-treated callus exhibited no detectable activity toward SA, suggesting that SA is not a precursor for syringyl lignin. However, all upstream steps of lignin biosynthesis (PAL activity, C4H transcription, 4CL activity toward FA) exhibited increased activity. These results suggest that SA may induce or enhance enzyme activities involved in syringyl lignin biosynthesis in poplar callus.

Key words Ferulic acid · Lignin biosynthesis · Poplar callus · Sinapic acid · Syringyl lignin

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Introduction

Lignin is essential for plants in the colonization of land, as it imparts mechanical strength and water impermeability, among other factors. It is composed mainly of guaiacyl and syringyl monomers in angiosperms. However, angiosperm lignins' monomeric composition vary according to the tissue and cell types and the environmental conditions.[1,2] Many enzymes are associated with lignin biosynthesis, and the biosynthetic route for syringyl lignin has been investigated extensively.[3,4]

Recent studies have suggested a new route for lignin biosynthesis. Conversion of guaiacyl nuclei to syringyl nuclei occurs through hydroxylation and methylation at the 5-position of the guaiacyl nucleus at the cinnamyl aldehyde or alcohol stage.[5–9] Enzyme kinetic studies using recombinant proteins and xylem protein extracts suggest that caffeic acid is a major precursor of coniferyl and sinapyl alcohols, but biosynthesis of lignin via ferulic acid (FA) and sinapic acid (SA) is under debate.[7,10] Evidence against SA-dependent syringyl lignin biosynthesis has been obtained through investigations showing low or undetectable 4-coumarate:coenzyme A ligase (4CL) activity with SA.[11–17] On the other hand, FA is a possible precursor for monolignols, as it is a good substrate for most reported 4CLs and was shown many years ago to be effectively incorporated into guaiacyl and syringyl subunits in lignin.[18–20]

Feeding of labeled precursors is useful when investigating precursor metabolism; however, the possibility exists that the subsequently observed metabolic route is an abnormal pathway induced by the exogenous precursors. Enzyme studies carried out in vitro are essential for kinetic characterization of metabolic enzymes and the effects of their inhibitors, but these studies are often performed with nonphysiological concentrations of enzyme, substrate, or inhibitors. In our feeding experiments,[21] tetradeuteroferulic acid (DFA) exogenously supplied to the poplar callus was effectively converted to both guaiacyl and syringyl lignin, suggesting that FA is a possible precursor of monolignol in the callus. When heptadeuterosinapic acid (DSA) was sup-

plied, it was not incorporated into syringyl lignin or guaiacyl lignin. However, yields of thioacidolysis product from syringyl lignin were markedly increased in DSA-treated callus. These results suggest that SA is not a precursor of sinapyl alcohol and syringyl lignin per se, although it may induce or enhance the biosynthesis of syringyl lignin in poplar callus.

In this study, to better interpret the results of our feeding experiments,[21] we examined several enzyme activities involved in lignin biosynthesis, such as phenylalanine ammonia-lyase (PAL), cinnamate:4-hydroxylase (C4H), 4CL, and cinnamyl alcohol dehydrogenase (CAD) activity in poplar callus. Substrate utilization of the callus 4CL well supports the findings of previous experiments.[21] The increased enzyme activity upstream (PAL, C4H, 4CL toward FA) in lignin biosynthesis also explains the increased syringyl lignin biosynthesis in poplar callus by exogenously supplied SA.

Materials and methods

Plant materials and administration of cinnamic acid derivatives to poplar callus

The poplar (*Populus alba* L.) callus used in this study was induced on Murashige and Skoog basal medium supplemented with 3% sucrose, 2,4-D 1.0 ppm, kinetin 0.5 ppm, and 0.8% agar. The callus was maintained on the same medium at 25°C in the dark.[22] FA and SA were individually dissolved in 1,4-dioxane/water (9:1, v/v) and added to the medium to obtain a final concentration of 0.5 g/l, where poplar calli were cultivated in 3 weeks.

4-Coumarate:CoA ligase assay

All steps were carried out at 4°C. Protein extraction was performed as described by Allina et al.[11] with some modifications. Poplar tissue was homogenized in liquid N_2 and mixed with 300 mM Tris-HCl buffer (pH 7.8) containing 14 mM 2-mercaptoethanol, 30% (v/v) glycerol, and Dowex AG1-X2 Resin (Bio-Rad; 0.1 g of resin/g of tissue in 2 ml of buffer). The mixture was rotated for 20 min, centrifuged, and filtered to remove the resin and debris.

The 4CL activity was assayed by spectrophotometric monitoring of the formation of coenzyme A (CoA) esters of various cinnamic acid derivatives, as described by Knobloch and Hahlbrock.[23] The 4CL reaction mixture contained the callus extract isolated as described above plus 5 mM ATP, 5 mM $MgCl_2$, 0.33 mM CoA, and 0.2 mM cinnamic acid derivative in a total volume 0.4 ml. Two blank (reference) mixtures lacking CoA and enzyme (callus extract) were used. Enzyme activity was measured by following the increase in absorbance at the absorption maximum of the appropriate CoA ester.[16] Enzyme activity was calculated using extinction coefficients of 21 000 (at 333 nm), 18 000 (at 346 nm), 19 000 (at 345 nm), 20 000 (at 356 nm), and 20 000 (at 352 nm) for *p*-coumaroyl CoA, caffeoyl CoA, feruloyl CoA, 5-hydroxyferuloyl CoA, and sinapoyl CoA, respectively.[16]

Phenylalanine ammonia-lyase assay

Protein extraction for PAL assays was performed by a method similar to that described above for 4CL, except that the extraction buffer contained 200 mM borate (pH 8.8), 14 mM 2-mercaptoethanol, and 10% Dowex AG1-X2. Enzyme reactions were carried out at 37°C and contained callus extract, 7.5 mM phenylalanine, and 100 mM borate buffer (pH 8.8). The formation of cinnamic acid as a result of PAL activity was monitored spectrophotometrically by following the increase in absorbance at 290 nm, according to the method of Tsutsumi and Sakai.[22]

Cinnamyl alcohol dehydrogenase asssay

For CAD assays, protein extraction was performed similarly, except that the extraction buffer contained 200 mM Tris-HCl (pH 7.5) and 20% glycerol. Enzyme reactions were carried out at 30°C in 0.4-ml reaction mixtures containing 100 mM KH_2PO_4/Na_2HPO_4 (pH 6.25), 100 μM substrate, 100 μM reduced nicotinamide adenine dinucleotide phosphate (NADPH), and 50 μl callus extract. The formation of cinnamaldehyde and the consumption of NADPH as a result of CAD activity were monitored spectrophotometrically by following the change in absorbance at 340 nm, as described in Yahiaoui et al.[24]

Northern blot analysis of cinnamate:4-hydroxylase mRNA

Total RNA (15 μg) was extracted from calli as described by Chomczynski and Sacchi,[25] electrophoresed on a 1% agarose gel containing 18% formaldehyde, and then transferred to a Hybond-H+ membrane (Amersham). The probe was a 1288-bp fragment of poplar H11 C4H cDNA[26] labeled with the Multiprime DNA labeling system (Amersham) using ^{32}P-labeled deoxycytidine triphosphate (^{32}P-dCTP). After probe hybridization, the membrane was washed with 0.5 × SSC, 0.1% sodium dodecyl sulfate (SDS) at 55°C.

Results and discussion

Incorporation of ferulic or sinapic acid and 4CL activity in callus

The importance of 4CL as a regulatory enzyme in the biosynthesis of plant phenolics has been reported (for reviews see Lee and Dougles[14] and references therein, Ehlting et al.,[12] and Hu et al.[13]). In another study[27] the lignin content of aspen (*Populus tremuloides* Michx.) was reduced to half that of the control plant as a result of anti-sense 4CL mRNA suppression, suggesting that 4CL is one of the major
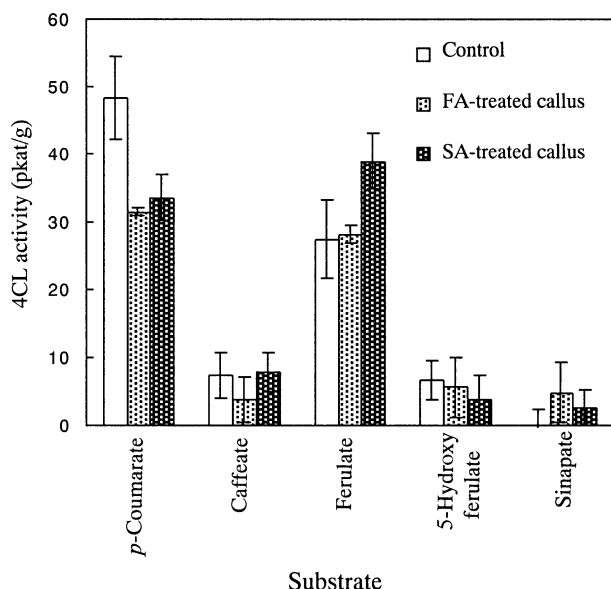
Fig. 1. 4-Coumarate:CoA ligase (*4CL*) activity in crude enzyme preparations from poplar callus as determined using cinnamic acid derivatives as substrates. Crude enzyme preparations from control, ferulic acid (*FA*)-treated and sinapic acid (*SA*)-treated callus were used. Substrate concentration and other conditions were kept constant for each set of assays, and each assay was performed in duplicate. Data shown are means of three individual experiments, and error bars indicate standard deviations. Because the spectrophotometric error associated with the data is large, activity levels of <10pkat/g cells are below the limit of reproducible detection

Table 1. Phenylalanine ammonia-lyase activity in extracts of poplar callus

Extract	PAL activity (pkat/g fresh callus)
Control	14.1 ± 2.7 (100%)[a]
FA-treated callus	12.6 ± 0.5 (89%)[a]
SA-treated callus	22.0 ± 4.6 (156%)[a]

PAL, phenylalanine ammonia-lyase; FA, ferulic acid; SA, sinapic acid
[a] The activity in extracts from control callus is taken as 100%

The lack of callus 4CL activity toward caffeic acid may partially contribute to the decreased synthesis of lignin in poplar callus compared to xylem cells. Considering the results of our feeding experiments[21] and 4CL assays together, it seems likely that FA rather than caffeic acid is a precursor for lignin biosynthesis in the callus.

PAL, C4H, and CAD activity in callus treated with ferulic or sinapic acid

Although exogenously supplied SA was not converted to callus lignin in our previous study,[21] it clearly induced de novo synthesis of syringyl lignin in the callus. Thus, we compared PAL activity, C4H transcription, and 4CL activity in the upstream metabolic steps of monolignol biosynthesis in control and FA- and SA-treated calli. PAL catalyzes the first metabolic step from primary metabolism into phenylpropanoid metabolism,[32] and the converted cinnamic acid is catalyzed to *p*-coumaric acid by C4H.[33,34] In this study, CAD activity was also assayed, using coniferyl aldehyde and sinapyl aldehyde as substrates, because this enzyme catalyzes the last step in biosynthesis of monolignols.

The PAL activity in SA-treated callus increased to 1.6 times that in the control callus (Table 1). This increase in PAL activity is consistent with the increase in lignin content observed for the SA-treated callus.[21] Northern blot analysis clearly indicated that C4H transcription was also increased by exogenously supplied SA (Fig. 2). The increase of PAL activity and C4H transcription in the first step of monolignol biosynthesis may explain the increase of carbon flow into lignin biosynthesis in SA-treated callus. In addition, 4CL activity toward FA in the SA-treated callus increased, but the activity toward caffeic acid remained unchanged compared to the control (Fig. 1). Assuming that FA is a precursor for callus lignin, this increase in 4CL activity is also in agreement with our assertion, and the three enzymes located upstream in the monolignol biosynthetic pathway were enhanced by exogenously supplied SA. Because enzymes (PAL, C4H, 4CL) in the initial step of monolignol biosynthesis are shared between the guaiacyl and syringyl monolignol pathways, the specific enhancement of syringyl lignin biosynthesis in SA-treated callus begs further explanation.

In callus, CAD activity toward sinapyl aldehyde, the last step in the syringyl monolignol-specific pathway,[10] was slightly decreased in SA-treated callus (Table 2), suggesting that the impact of CAD on lignin biosynthesis may not be
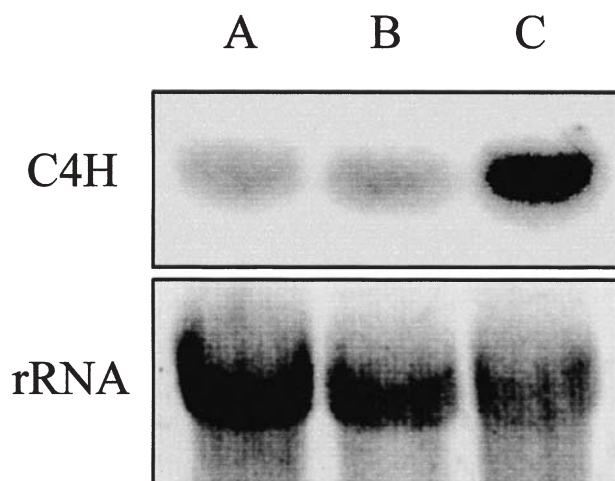
regulatory enzymes in lignin biosynthesis. Moreover, recent studies proposed that caffeic acid is the sole precursor in lignin biosynthesis.[7,10,28,29] However, in poplar callus we showed that FA, but not SA, was efficiently incorporated into guaiacyl and syringyl lignin.[21] Therefore, in this study the substrate utilization of 4CLs prepared from the control and SA- and FA-treated callus was investigated with five cinnamic acid derivatives as substrates.

The crude callus 4CL extract from SA-treated callus exhibited a clear preference for *p*-coumaric acid and FA and was almost inactive toward caffeic acid, 5-hydroxyferulic acid, and SA substrates (Fig. 1). Therefore, conversion of SA to sinapyl alcohol clearly does not occur in poplar callus in vivo. The lack of 4CL activity toward SA is consistent with other reports[11–17] and supports our results that exogenously supplied heptadeutero-SA is not converted to sinapyl alcohol.[21]

The activity of the crude 4CL extract from FA-treated callus had a strong substrate preference for *p*-coumaric acid and FA, as well as for control and SA-treated callus (Fig. 1). This and the results of FA incorporation in our initial paper of this series[21] indicate that the exogenously supplied FA is converted to feruloyl CoA and then to coniferyl and sinapyl alcohols in poplar callus. Although many 4CL enzymes utilize caffeic acid as a substrate,[11,30,31] in our study the callus 4CL showed as little activity toward caffeic acid as SA. Accordingly, just as supplied SA was not converted to callus lignin because of its low 4CL activity, conversion of caffeic acid to caffeoyl CoA would be problematic in the callus.

Fig. 2. Northern blot analysis of cinnamate:4-hydroxylase (*C4H*) mRNA levels from control (*A*), FA-treated (*B*), and SA-treated (*C*) poplar callus. **Top** Total RNA (15 μg) from each callus was electrophoresed on formaldehyde agarose gels, transferred to nylon membranes, and hybridized to a C4H cDNA probe. **Bottom** Ethidium bromide staining of the 28S rRNA band confirms equal loading of lanes

Table 2. Cinnamyl alcohol dehydrogenase activity in extracts of poplar callus

Extract	CAD activity (nkat/g fresh callus)	
	Coniferyl aldehyde	Sinapyl aldehyde
Control	1.92 ± 0.30 (100%)	2.56 ± 0.25 (100%)
FA-treated callus	2.15 ± 0.38 (113%)	2.98 ± 0.20 (116%)
SA-treated callus	1.64 ± 0.27 (86%)	1.86 ± 0.38 (73%)

CAD, cinnamyl alcohol dehydrogenase

significant. In fact, down-regulation of CAD in poplar does not significantly change the lignin content or the monomeric composition.[35]

Exogenously supplied FA was converted to guaiacyl and syringyl lignin,[21] but the distinct changes in the enzyme activities investigated here were not observed in the FA-treated callus. This observation suggests that the enzymes required for syringyl monolignol biosynthesis may be sufficient for processing in the callus.

In the context of regulating the syringyl/guaiacyl units (S/G) ratio in lignin, some routes for conversion of guaiacyl nuclei to syringyl nuclei at different stages, such as cinnamyl aldehyde or cinnamyl alcohol, have been proposed.[6–10,28] Thus, there are many possibilities for the mechanisms responsible for the increased of syringyl lignin due to exogenous SA.

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