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NOTE

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Generation of transgenic hybrid aspen that express a bacterial gene for feruloyl-CoA hydratase/lyase (*FerB*), which is involved in lignin degradation in *Sphingomonas paucimobilis* SYK-6

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Abstract Transgenic hybrid aspens that expressed a bacterial gene for an enzyme involved in the degradation of hydroxycinnamates, feruloyl-CoA hydratase/lyase (*FerB*), were produced by *Agrobacterium*-mediated transformation. The gene was isolated from the bacterium *Sphingomonas paucimobilis* SYK-6, which is able to degrade a wide variety of monomeric and dimeric lignin-related compounds. The introduced gene was expressed and FerB (vanillin-forming) activity was detected in cell-free extracts obtained from most of the plants. There were no detectable differences between the transgenic plants and wild-type plants, except in one of the transformants 33 that had chlorotic leaves and a lower growth rate. The results of reverse transcription-polymerase chain reaction analysis of endogenous genes for phenylpropanoid biosynthesis suggested that expression of the *FerB* transgene did not have a significant effect on the expression of endogenous genes in transformants at an early stage of growth, namely in 2-month-old plantlets, even though levels of expression of genes for 4-coumarate:CoA ligase and ferulate 5-hydroxylase were higher in several of the FerB transformants than in the wild-type plants. Our data indicate that genes derived from bacteria can be expressed in woody plants and suggest a new method for manipulating phenylpropanoid biosynthesis and for producing transgenic woody plants with enhanced economic value.

Key words Lignin · Phenylpropanoid · *Sphingomonas* · Transgenic aspen

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Introduction

Phenolic compounds that are synthesized via the phenylpropanoid pathway, such as lignin, lignan, flavonoids, and benzoates, play important roles in plant growth and development and in the defense against pathogens. Lignin, in particular, contributes to the mechanical support of cell walls, to water transport, and to resistance to attacks by microorganisms. The complexity of the molecular structure of lignin, an organic polymer, prevents most microorganisms from degrading it easily. However, some fungi and bacteria can degrade native and low molecular weight lignins, respectively. For example, the soil bacterium *Sphingomonas paucimobilis* SYK-6 produces enzymes that can degrade various monomeric and dimeric lignin-related compounds, such as beta-aryl ether-, biphenyl-, phenylcoumarane-, and pinoresinol-type molecules.[1,2] Some of the genes for these enzymes have already been cloned and characterized.[2]

Several bacterial genes for enzymes that are involved in phenolic metabolism have been introduced into plant genomes, and transgenic plants that express these genes have been generated. For example, the introduction of *nahG*, which encodes a bacterial salicylate dehydrogenase, into tobacco yielded transgenic plants with lower levels of salicylate and helped to clarify the role of salicylate in plants.[3] The *ubiC* gene for chorismate pyruvate-lyase was also successfully expressed in tobacco, and the transgenic plants were able to convert chorismate to 4-hydroxybenzoate in both the cytosol and plastids, even though this conversion does not occur in the cytosol of wild-type tobacco.[4] This

observation suggested that chorismate is present not only in plastids but also in the cytosol of plant cells. In another study, a gene for enoyl-CoA hydratase/lyase (HCHL), isolated from *Pseudomonas fluorescens*, was able to moderate the phenylpropanoid pathway in transgenic plants and alter the levels of soluble phenolic compounds relative to those in wild-type plants.[5,6] The results from these studies indicate that expression of bacterial genes in plants can contribute both to knowledge of the metabolism of phenolics in plants, and to the production of transgenic plants with novel and economically important characteristics.

Modification of lignin in the secondary walls of plant cells can improve the utilization of the lignocellulosic biomass produced by plants.[7–9] Recent studies indicate that the lignin content and/or composition of cell walls can be manipulated by regulation of the expression of endogenous genes for phenylpropanoid- and lignin-biosynthetic enzymes.[10,11] It has been suggested that some of the phenolic compounds in the lignin degradation pathway of *S. paucimobilis* SYK-6, such as hydroxybenzoates, hydroxybenzaldehydes, hydroxycinnamates, and hydroxycinnamoyl-CoA esters, are important as intermediates in the phenylpropanoid- and lignin-biosynthetic pathways. Thus, it might be possible to modify the biosynthesis of phenylpropanoids via the expression of genes for lignin-degrading enzymes from *S. paucimobilis* SYK-6 in transgenic plants (Fig. 1). This report describes a feasibility study in which we examined this possibility, using one of the genes, *FerB*, that is involved in the metabolism of hydroxycinnamoyl-CoA esters in this bacterium.

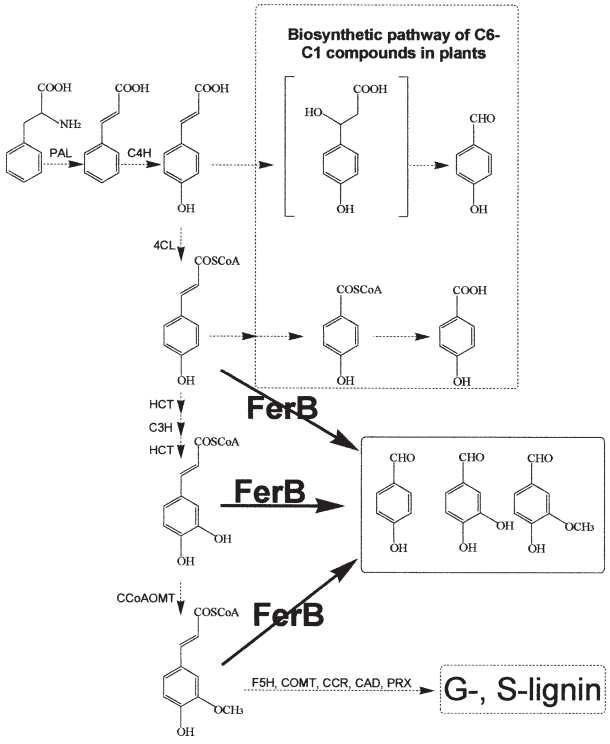

Fig. 1. Simplified putative pathways for the biosynthesis of lignin and C6-C1 compounds. Enzymes involved in the various pathways are: *PAL*, phenylalanine ammonia-lyase; *C4H*, cinnamate 4-hydroxylase; *4CL*, 4-coumarate:CoA ligase; *HCT*, hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyltransferase; *C3H*, 4-coumarate 3-hydroxylase; *CCoAOMT*, caffeoyl-CoA *O*-methyltransferase; *F5H*, ferulate 5-hydroxylase; *COMT*, caffeate/5-hydroxyferulate *O*-methyltransferase; *CCR*, cinnamoyl-CoA reductase; *CAD*, cinnamyl alcohol dehydrogenase; *PRX*, anionic peroxidase; *FerB*, feruloyl-CoA hydratase/lyase

Materials and methods

Plant material

Transgenic plants were generated from young plantlets of hybrid aspen (*Populus sieboldii* × *P. gradientata*) that had been grown under sterile conditions by *Agrobacterium*-mediated transformation. Plantlets were cultured on modified Murashige-Skoog (MS) medium supplemented with 0.05 mg/l indole-3- butyric acid (IBA) in glass tubes (40 mm i.d. × 130 mm).[12]

Construction of plasmids and transformation of hybrid aspen

The amino acid coding region of the *FerB* gene (860 bp; Accession number AB072376) was amplified by polymerase chain reaction (PCR) using plasmid pKHR126[13] as the template. The fragment of interest was then cloned in the pCR2.1 vector (Invitrogen, Carlsbad, CA). For construction of the expression vector for plant transformation, we inserted the *FerB* fragment between the 35S promoter of the cauliflower mosaic virus and an octopine synthase terminator in the binary vector pART27.[14] The resultant plasmid, designated pTRFerB, was introduced into *Agrobacterium tumefaciens* LBA4404 for the transformations.

Plantlets of hybrid aspen were transformed as described by Ebinuma et al.,[12] except that we used modified MS medium supplemented with 0.5 mg/l *t*-zeatin and 100 mg/l kanamycin for the regeneration of transgenic shoots from sections of stem tissue. Regenerated shoots were rooted on MS medium supplemented with 0.05 mg/l IBA and 100 mg/l kanamycin. The resultant young plants were cultured on the same medium in glass tubes under sterile conditions.

Assay of FerB activity in cell-free extracts of transgenic plants

Cell-free extracts were prepared from homogenized leaf tissue (ca. 200 mg fresh weight) from individual 2-month-old transgenic plants. Homogenates were prepared in an extraction buffer [100 mM Tris-HCl (pH 7.5), 50 μM 2-mercaptoethanol] on ice. After centrifugation of the homogenates, each supernatant was used for the assay of enzymatic activity after overnight dialysis against the extraction buffer. The FerB (vanillin-forming) activity in each extract was detected as follows. In the first step of the assay, the substrate for the reaction, feruloyl-CoA, was synthesized enzymatically by recombinant feruloyl-CoA synthase

(FerA), which had been prepared from *Escherichia coli* that had been transformed with the *FerA* gene from *S. paucimobilis* SYK-6.[13] For the synthesis of feruloyl-CoA, 2 ml of a buffer solution comprising 100 mM Tris-HCl (pH 7.5), 2.5 mM adenosine triphosphate (ATP), 200 μM CoA, 500 μM ferulate, and 2.5 mM $MgSO_4$ was incubated at 30°C for 1 h. After the synthesis of feruloyl-CoA, the cell-free extract (300 μg of protein) from a transgenic plant was added to the reaction mixture which was then incubated at 30°C for 1 h. The reaction was stopped by lowering the pH to 2 by the addition of HCl, and the resultant mixture was extracted three times with dimethylether. The volume of the organic fraction was reduced by evaporation to approximately 30 μl, of which 10 μl was subjected to thin-layer chromatography (TLC) with a mixture of chloroform, ethyl acetate, and formic acid (in the ratio 10 : 8 : 1) as the mobile phase.

Analysis by reverse transcription-PCR

Using reverse transcription and PCR (RT-PCR), we examined the expression of the genes for FerB, phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), ferulate 5-hydroxylase (F5H), caffeate/5-hydroxyferulate *O*-methyltransferase (COMT), cinnamyl alcohol dehydrogenase (CAD), and anionic peroxidase (PRX) using total RNA isolated from stem tissues of individual transformants. The design of each pair of primers was based on the nucleotide sequence of each respective gene as reported by our group and others. We also analyzed the expression of rRNA gene in each transformant by RT-PCR, using commercial primers (Classic II 18S Internal Standards; Ambion, Austin, TX), for subsequent normalization of the expression of other genes. In the analysis, first-strand cDNA was synthesized from total RNA using a random primer (for analysis of rRNA gene expression) or oligo-dT primer (for other gene expressions), and used as templates for PCR. The nucleotide sequences of the forward and reverse primers used in the amplification of PAL, C4H, 4CL, F5H, COMT, CAD, PRX, and FerB are as follows: PAL-F, 5′-AAGCAAGG CGGAGAGCTTCAAAAG-3′; PAL-R, 5′-CTTGCAT AACTTCAGCAAAGATTG-3′; C4H-F, 5′-CTTTACAT TGTTGAGAACATCAAC-3′; C4H-R, 5′-CTGAGGTG TCGATCTTTGACTGTC-3′; 4CL-F, 5′-ACTGTAGTCA GGAATGCAGAGATG-3′; 4CL-R, 5′-TCTTTCAGATT CTTCCTCAGGATC-3′; F5H-F, 5′-ACGAATCAGATG ATTTACAAAA-3′; F5H-R, 5′-GGAATAAATTCGAA GTGATTCC-3′; COMT-F, 5′-ACTGGAGCTGTCGTT AACACCATC-3′; COMT-R, 5′-TAGGCCTTCTTGCGG AATTCAATG-3′; CAD-F, 5′-TGGACTGAAACAGAG TGGGCTAAG-3′; CAD-R, 5′-TACCAGCAACATCGA CAACGAATC-3′; PRX-F, 5′-TCTTGCCATTGCAGCT GAACAGTC-3′; PRX-R, 5′-CAATGGTATCGGCAC CTGTAGTAG-3′; FerB1, 5′-GCATGTCCGAGGAGGA TAAGC-3′; and FerB2, 5′-TCATCAGACCTCGGTC TTGG-3′.

To distinguish amplified DNA fragments derived from first-strand cDNA from those derived from contaminating genomic DNA in total RNA, we designed pairs of primers for the amplification of intron-containing fragments. In addition, to check that the amplification by PCR was linear, each of the first-strand cDNAs derived from the total RNA (34 ng equivalent) was amplified with the appropriate primers by PCR using at least four different numbers of amplification cycles (from 17 to 35 cycles). For example, PCR was performed at 29, 31, 33, and 35 cycles in the case of the PAL gene; after checking that the amplification at these cycles was in the linear range by electrophoresis of the amplified PAL DNA, one set of the amplified DNA samples that reacted at 33 cycles was used for quantitative analysis of fluorescence intensity. After the separation of the amplified DNA by electrophoresis, the DNA on the gel was stained with Nucleic Acid Stain (Vista Green; Amersham Biosciences, Tokyo, Japan), and the fluorescence intensity of the stained DNA was quantified with an image analyzer (FluorImager 595; Amersham Biosciences) equipped with software for quantification (IMAGE Quant; Amersham Biosciences). The fluorescence intensities of the amplified DNA of eight genes (PAL, C4H, 4CL, F5H, COMT, CAD, PRXA3, and FerB) were normalized to that from amplified DNA from rRNA in the same plant.

Results

Phenotypes of transgenic aspen plantlets that harbored a *FerB* transgene

In addition to ten control transformants that harbored a kanamycin resistance gene exclusively, we obtained a total of 33 independent transformed lines that harbored the *FerB* transgene cassette. The presence of the transgene in each plant line was confirmed by PCR (data not shown). Each transformant was maintained in a small glass bottle after rooting and then propagated clonally by transferring individual shoots into rooting medium.

We examined FerB activity in 23 plants randomly selected from among our 33 FerB transformants, as described in the Materials and methods section. Seventeen of the tested transformants appeared to exhibit FerB activity even though our technique for measuring FerB activity was not quantitatively exact. We chose nine of these FerB transformants for further analysis after confirming the reproducibility of the detection of FerB activity in each plant (Fig. 2). We expected that some vanillin-forming activity would also be detectable in wild-type aspen plants because a large amount of *p*-hydroxybenzoate and small amounts of vanillin and syringaldehydes are associated with cell wall fractions from *Populus* trees,[15] suggesting the existence in wild-type plants of the synthesis of C6-C1 compounds from C6-C3 compounds, such as hydroxycinnamates or hydroxycinnamoyl-CoAs. Although we detected no activity in our extracts of wild-type plants (Fig. 2), we cannot exclude the possibility described above because we did observe a faint signal after analysis by TLC of the cell-free extract from a wild-type plant after we extended the reac-

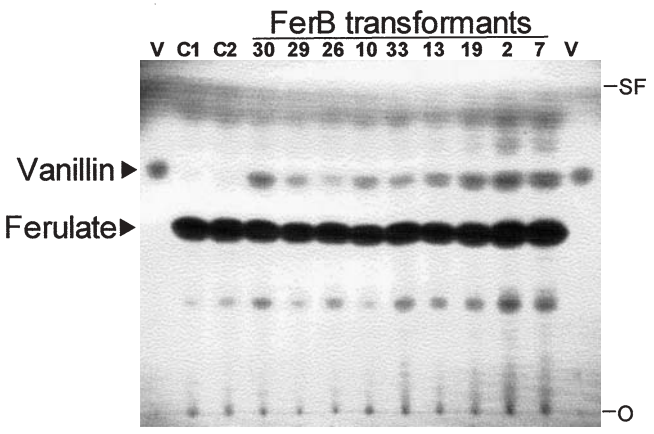

Fig. 2. Analysis of FerB (vanillin-forming) activity by thin-layer chromatography (TLC). Products of reactions with cell-free extracts derived from wild-type plants (*C1* and *C2*) and FerB transformants were analyzed after acidification and subsequent extraction of the reaction mixtures. Each sample and authentic vanillin (*V*) were subjected to TLC. In addition to vanillin that was produced from feruloyl-CoA ester by FerB in cell-free extracts, some of the ferulate that had been added to the reaction mixture for preparation of the CoA ester was also detected. The photograph was taken under ultraviolet light. *O*, origin; *SF*, solvent front

tion time to 24 h (data not shown). We used gas chromatography-mass spectrometry (GC-MS) to confirm the production of vanillin from feruloyl-CoA in the reaction mixture of cell-free extracts prepared from some of the FerB transformants (data not shown).

The morphology of most of the FerB transformants was similar to that of wild-type plants transformed with the empty vector, but one transformant line (33) had unusually slender leaves that exhibited chimeric chlorosis (Fig. 3A). This abnormal phenotype was also maintained in all the clonally propagated plantlets derived from this transformant. In addition, the transformant 33 exhibited stunted growth and decreased root elongation during further growth.

For a preliminary assessment of differences in lignin deposition between the wild-type plants and the FerB transformants, we stained hand-cut sections of stems with the Wiesner or Mäule reagents (Fig. 3B-E). There were no significant differences from the wild-type plants in terms of the staining and arrangement of cells among all of the FerB transformants tested, suggesting that the lignin content and composition of plants of this age (2 months) is not influenced to any major extent by the introduction of the *FerB* transgene.

RT-PCR analysis of the expression of FerB and several phenylpropanoid-biosynthetic genes

To confirm the expression of the *FerB* gene in the transformants and to examine its effect on those of phenylpropanoid-biosynthetic genes, we used RT-PCR to analyze total RNA prepared from stem tissues of the wild-type plants and those of FerB transformants. As indicated

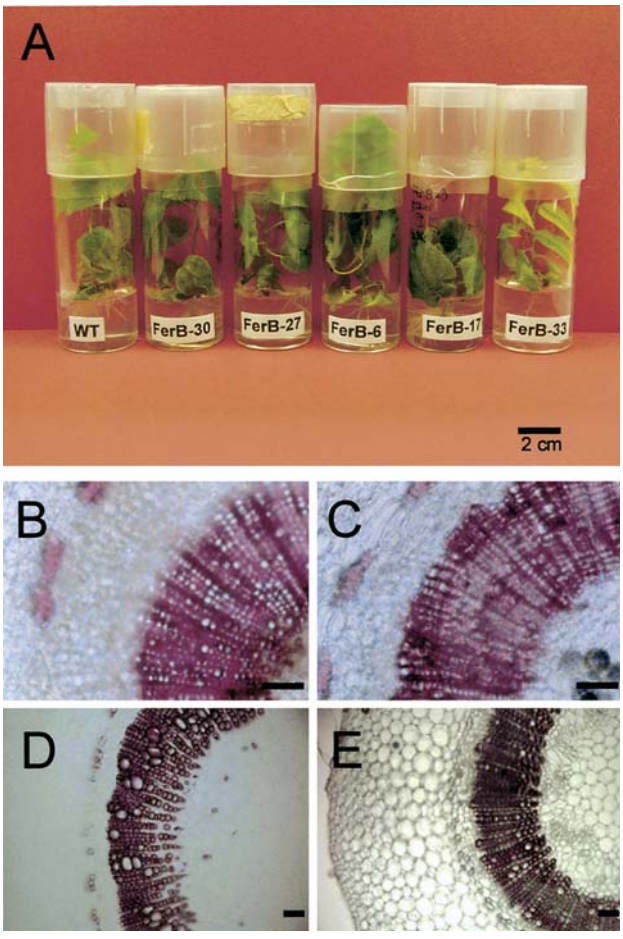

Fig. 3A–E. The phenotypes of young FerB transformants. **A** Morphologies of wild-type (*WT*) transgenic plant (*left*) and FerB transformants. **B** Section of a wild-type plant stained with phloroglucinol-HCl. **C** Section of an FerB transformant stained with phloroglucinol-HCl (transformant 30). **D** Section of a wild-type plant stained with Wiesner reagent. **E** Section of an FerB transformant stained with Wiesner reagent (transformant 30). All sections were cut from stems. *Bars* **A** 2 cm; **B–E** 50 μm

in Fig. 4, all of the FerB transformants tested expressed the *FerB* transgene to a greater or lesser extent, confirming that the vanillin-forming activity detected in the cell-free extracts was derived from the expression of the *FerB*. As in the case of the *FerB* transgene, the levels of expression of all of seven other genes that we analyzed varied among the wild-type plants and the FerB transformants. To compare the levels of gene expression semiquantitatively between the wild type and the FerB transformants, the level of each gene expression was assessed as the fluorescence intensity of amplified DNA in RT-PCR. Each value of fluorescence intensity was normalized to that of amplified DNA from rRNA from the same plant. Our results indicated that although the fluorescence intensity of amplified DNA from the *FerB* transcript in each transformant was correlated with those of other genes with correlation coefficients (R^2 values) that varied from 0.075 to 0.86, the variations among the values of most of the analyzed genes in the FerB transformants were similar to those in the control

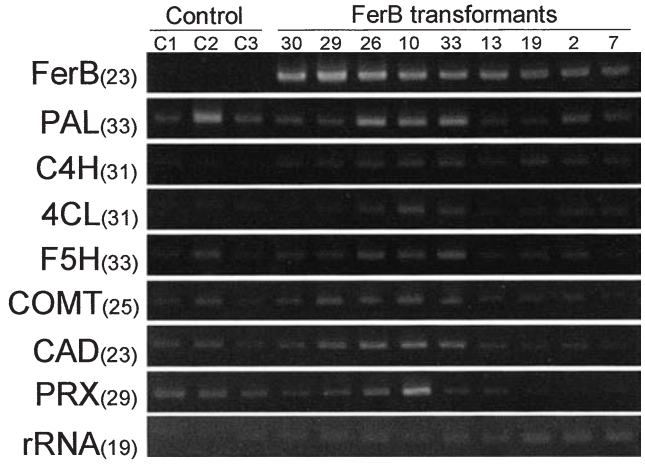

Fig. 4. Analysis by reverse transcription-polymerase chain reaction (RT-PCR) of the expression of *FerB* and phenylpropanoid-biosynthetic genes. See text for details and Fig. 1 for abbreviations. *Numbers in parentheses* indicate the number of PCR cycles used in the amplification of each gene transcript

transformants. Moreover, even though our present data were not analyzed statistically due to the number of samples being insufficient, it is worth noting that the fluorescence intensity of the amplified DNA from *4CL* and *F5H* transcripts in transformants 10, 26, 30, and 33 were, in some cases, close to two-fold higher than those in the control plants (4CL, fluorescence intensity was 2.2–4.6 $\times$ 10^5 for control plants and 6.0–8.6 $\times$ 10^5 for transformants 10, 26, 30, and 33; F5H, fluorescence intensity was 1.7–4.7 $\times$ 10^5 for control plants and 8.8–9.6 $\times$ 10^5 for transformants 10, 26, 30, and 33). No amplified products derived from contaminating genomic DNA were detected in any of the plants after the PCR analysis.

Discussion

Recent studies of the biosynthetic pathway for lignin have reinforced the hypothesis that hydroxycinnamoyl thioesters, such as *p*-coumaroyl, caffeoyl-, and feruloyl-CoA, play prominent roles in the hydroxylation and methylation steps that are required for the synthesis of guaiacyl and syringyl moieties.[16,17] In addition, it is noteworthy that hydroxybenzoates and hydroxybenzaldehydes, which are probably synthesized from hydroxycinnamoyl-CoA and/or hydroxycinnamates, bind to cell wall fractions from *Populus* trees via ester and/or ether bonds, and they account for approximately 9% of the dry weight of wood.[15,18] These data suggest that bypassing the phenylpropanoid pathway using the *FerB* transgene at the step that involves hydroxycinnamoyl-CoA esters might be useful as a technique for the biotechnological modification of lignin and related phenolic compounds that are incorporated into the secondary cell walls of *Populus* plants. In this study, we confirmed the expression of the *FerB* transgene and detected FerB activity in our transformants. To the best of our knowledge, this is the first report of the production of transgenic woody plants that harbor a bacterial gene for a lignin-degrading enzyme.

There have been reports of the introduction of a gene for HCHL, a homolog of the *FerB* gene in *Pseudomonas fluorescens*, into tobacco and *Datura stramonium* with resultant modification of soluble phenolics in transgenic tobacco plants and hairy roots of *D. stramonium*.[5,6] In these transgenic lines, soluble phenolic compounds derived from hydroxycinnamoyl-CoA esters, such as glucosides and glucose esters of hydroxybenzoate, hydroxybenzyl alcohol, and vanillate, accumulated at high levels in leaves, flowers, seed capsules, and hairy roots. In contrast, levels of 3- and 4-caffeoylquinates, chlorogenate, and rutin were apparently reduced in the leaves of the transgenic tobacco plants. Stunting and leaf chlorosis, which we also observed in our transformant 33, were typical of the transgenic tobacco plants with the highest HCHL activity. Only plants with high levels of expression of the *HCHL* gene exhibited this abnormal phenotype, and with plants exhibiting lower levels of expression appearing normal.[5] The reason for the abnormal phenotype of transformant 33 is unclear, but it was probably due to expression of the *FerB* transgene because we have not seen this type of phenotypic change in hybrid aspen in our previous studies.

Our colorimetric analyses of hand-cut sections of FerB transformants failed to reveal any modification of lignin in our transformants. Mitra et al.[6] suggested, in their preliminary analysis, that there was a decrease in lignin content in hairy roots of *D. stramonium* that harbored the *HCHL* transgene. Thus, it may be possible to engineer changes in lignin biosynthesis using the *HCHL* transgene. Likewise it should be possible to modify phenolic compounds via expression of the *FerB* transgene in aspen plants, even though the phenolic compounds in our transgenic plants remain to be examined. FerB, unlike HCHL, can recognize sinapoyl-CoA ester as a substrate.[13,19] Thus, the activity of FerB can be expected to generate novel phenotypes in transgenic aspen via reactions that do not occur in plants with the *HCHL* transgene.

In our transformants, the introduction of the *FerB* transgene into the genome did not lead to apparent stimulation or suppression of the expression of genes for phenylpropanoid-biosynthetic enzymes. In contrast, in the leaves of tobacco plants that harbored the *HCHL* gene, the *PAL*, *C4H*, and *4CL* genes were activated.[5] The difference between these transgenic lines might have been due to the differences between plant species (tobacco and aspen) and the tissues (leaf and stem) used in the experiments, to differences in substrate specificity and/or kinetic parameters between FerB and HCHL proteins, and/or to the low level of the expression of the *FerB* transgene, which might have failed to influence the expressions of other genes in our transformants. We do not know which explanation is valid, but our preliminary analysis indicates that the *FerB* transgene can increase the levels of soluble phenolic compounds such as hydroxybenzoate derivatives in transgenic tobacco plants,[20] and thus the *FerB* gene might also alter levels of soluble phenolic compounds in transgenic plants.

Currently, our transformed plants are too small to allow definitive analysis of levels of soluble phenolic compounds and lignin. In addition, the content and compositions of lignin, and expressions of the genes for phenylpropanoid-biosynthetic enzymes may change with developmental stage.[21] In future studies, we will evaluate the effects of expression of *FerB* on levels of soluble phenolic compounds and lignin biosynthesis in older transgenic hybrid aspen plants.

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