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Regulation of Ferulate-5-Hydroxylase Expression in Arabidopsis in the Context of Sinapate Ester Biosynthesis[1]

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Sinapic acid is an intermediate in syringyl lignin biosynthesis in angiosperms, and in some taxa serves as a precursor for soluble secondary metabolites. The biosynthesis and accumulation of the sinapate esters sinapoylglucose, sinapoylmalate, and sinapoylcholine are developmentally regulated in Arabidopsis and other members of the Brassicaceae. The *FAH1* locus of Arabidopsis encodes the enzyme ferulate-5-hydroxylase (F5H), which catalyzes the rate-limiting step in syringyl lignin biosynthesis and is required for the production of sinapate esters. Here we show that *F5H* expression parallels sinapate ester accumulation in developing siliques and seedlings, but is not rate limiting for their biosynthesis. RNA gel-blot analysis indicated that the tissue-specific and developmentally regulated expression of *F5H* mRNA is distinct from that of other phenylpropanoid genes. Efforts to identify constructs capable of complementing the sinapate ester-deficient phenotype of *fah1* mutants demonstrated that *F5H* expression in leaves is dependent on sequences 3′ of the *F5H* coding region. In contrast, the positive regulatory function of the downstream region is not required for *F5H* transcript or sinapoylcholine accumulation in embryos.

Many investigations of plant metabolic pathways, gene regulation, and DNA transposition have exploited the dispensable nature of phenylpropanoid compounds. Most of these efforts have focused on phlobaphenes and anthocyanins because these conspicuous pathway end products have greatly facilitated genetic analyses. These investigations have resulted in the isolation and characterization of genes encoding enzymes and transcription factors required for the accumulation of these secondary metabolites (for review, see Dooner et al., 1991). In Arabidopsis phenylpropanoid metabolism gives rise to flavonoids, lignin, and sinapic acid esters. Mutants of Arabidopsis that are altered in flavonoid biosynthesis are collectively known as *transparent testa* mutants because these mutations decrease or eliminate the flavonoid-based condensed tannins that pigment the seed coat. Some of these loci have been shown to encode biosynthetic enzymes and others encode regulatory proteins (Koornneef, 1990; Shirley et al., 1995). Although flavonoid biosynthesis in Arabidopsis has been studied extensively at the genetic and molecular levels, much less is known about the genes involved in the biosynthesis of sinapic acid esters. Because these compounds are dispensable under laboratory conditions (Chapple et al., 1992), they provide additional targets for the genetic analysis of phenylpropanoid metabolism.

Arabidopsis and other members of the Brassicaceae accumulate three major sinapic acid esters, sinapoylglucose, sinapoylcholine, and sinapoylmalate (Fig. 1) (Bouchereau et al., 1991; Chapple et al., 1992), and the relative abundance of each of these compounds is regulated developmentally during the plant's life cycle (Strack, 1977; Mock et al., 1992; Lorenzen et al., 1996). Leaves contain only sinapoylmalate, whereas seeds accumulate primarily sinapoylcholine and smaller amounts of sinapoylglucose. During seed development de novo synthesis of sinapic acid leads to the production of sinapoylcholine. Through a series of interconversion reactions that are initiated upon imbibition, seed sinapoylcholine reserves provide the phenylpropanoid moiety for the synthesis of sinapoylmalate in expanding cotyledons. As seeds germinate, sinapoylcholine is hydrolyzed to yield sinapic acid, which is then re-esterified by sinapic acid:UDPG sinapoyltransferase to form sinapoylglucose. Sinapoylglucose is subsequently converted to sinapoylmalate by the activity of sinapoylglucose:malate sinapoyltransferase (Strack, 1982; Lorenzen et al., 1996). These interconversions are complete at approximately d 6 of seedling development, when de novo synthesis of sinapic acid contributes to the accumulation of sinapoylmalate in developing leaves.

Sinapate esters can be visualized by their blue fluorescence under UV light both in vivo and after TLC separation. The ease with which these compounds can be detected has facilitated the isolation of mutants defective in sinapate ester synthesis. These mutants have provided insights into the biological function of sinapate esters, and have enabled the isolation of genes involved in their synthesis. The best studied of these mutants is the sinapoylmalate-deficient *fah1* mutant (Chapple et al., 1992). Experiments with *fah1* demonstrated that sinapoylmalate is an important UV-B sunscreen in Arabidopsis (Landry et al., 1995), and cloning

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Abbreviations: 4CL, 4-hydroxycinnamoyl CoA ligase; CaMV, cauliflower mosaic virus; C4H, cinnamate-4-hydroxylase; F5H, ferulate-5-hydroxylase; OMT, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase; PAL, Phe ammonia-lyase; UDPG, UDP-Glc.

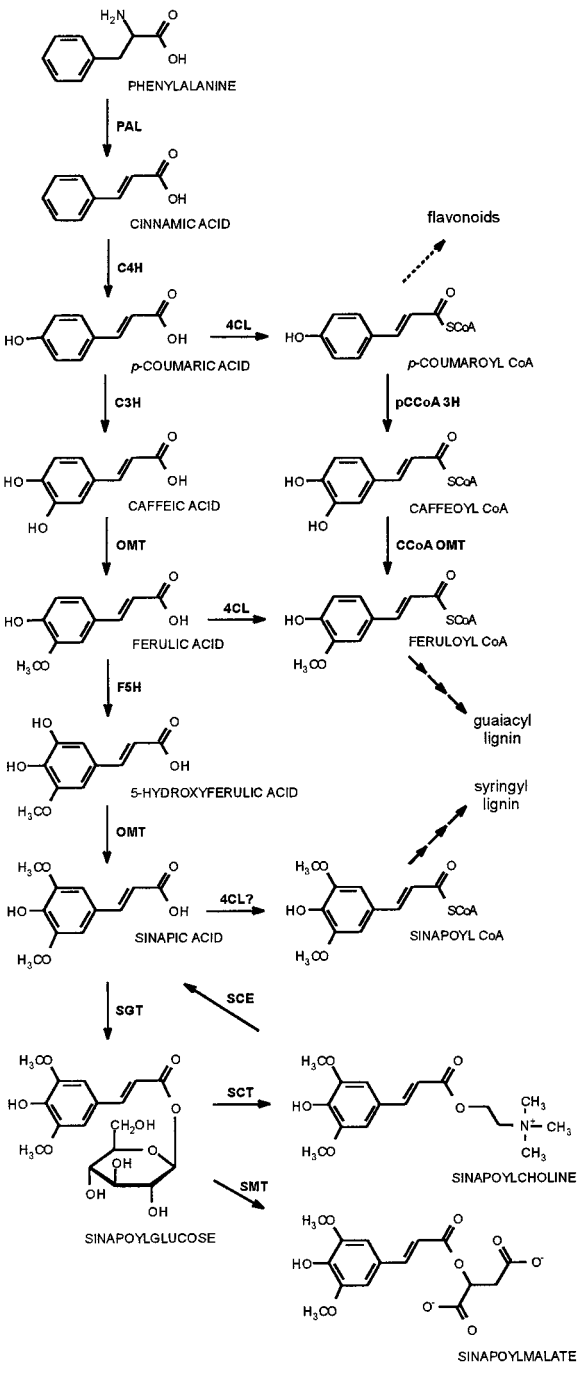

Figure 1. The phenylpropanoid pathway and the pathways leading to sinapate esters in Arabidopsis. CCoA OMT, Caffeoyl CoA *O*-methyltransferase; C3H, *p*-coumarate-3-hydroxylase; pCCoA 3H, *p*-coumaroyl CoA-3-hydroxylase; SCE, sinapoylcholinesterase; SCT, sinapoylglucose:choline sinapoyltransferase; SGT, sinapic acid: UDPG sinapoyltransferase; SMT, sinapoylglucose:malate sinapoyltransferase. The enzyme catalyzing the step from sinapic acid to sinapoyl CoA is shown as "4CL?" to reflect the uncertainty surrounding the identity of the protein involved.

of the *FAH1* gene revealed that it encodes F5H, a Cyt P450-dependent monooxygenase required for the synthesis of sinapate esters and sinapic acid-derived syringyl lignin (Meyer et al., 1996). It has since been shown that F5H catalyzes the rate-limiting step in syringyl lignin biosynthesis, and that its expression determines the monomer composition of the lignin in xylem and sclerified parenchyma (Meyer et al., 1998). Arabidopsis xylem cell walls contain only ferulic acid-derived guaiacyl lignin, whereas the interfascicular parenchyma of the rachis deposits syringyl lignin. When transformed with F5H ectopic-overexpression constructs, plants deposit syringyl-rich lignin in all cells that normally lignify, indicating that F5H is an important regulatory site for hydroxycinnamic acid production, at least with respect to lignin biosynthesis.

We investigated *F5H* expression in Arabidopsis in the context of sinapate ester biosynthesis. These experiments indicate that *F5H* transcript accumulation is regulated in a manner distinct from that of other phenylpropanoid genes. Furthermore, *F5H* expression in leaves is dependent on a regulatory domain that is located 3′ of the *F5H* stop codon, whereas its expression in embryos is independent of this downstream element. Although the pattern of *F5H* expression is consistent with a role for *F5H* in the determination of sinapate ester content, overexpression of *F5H* does not alter the temporal or tissue-specific regulation of sinapate ester accumulation. Thus, although F5H catalyzes the rate-limiting step in syringyl lignin biosynthesis, these findings cannot be extrapolated to imply a regulatory role for F5H in the biosynthesis of all sinapic acid-derived metabolites.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis plants were grown under a 16-h light/8-h dark photoperiod in potting mix (ProMix, Premier Horticulture, Red Hill, PA) at 22°C. For the growth of seedlings under axenic conditions, seeds were surface-sterilized in 30% (v/v) commercial bleach containing 0.07% (v/v) Triton X-100, rinsed, and sown on plates containing modified Murashige-Skoog medium lacking ammonium nitrate, as described previously (Lorenzen et al., 1996).

RNA Analysis

For the isolation of RNA, plant tissues were harvested, frozen in liquid nitrogen, and stored at −70°C until ready for extraction. Total RNA was isolated as described previously (Goldsbrough and Cullis, 1981). Samples were electrophoretically separated, transferred to membranes (Hybond N^+, Amersham), hybridized at 65°C, washed, and exposed to film. DNA probes for the Arabidopsis genes encoding 4CL (Lee et al., 1995), C4H (Bell-Lelong et al., 1997), F5H (Meyer et al., 1996), OMT (Arabidopsis expressed sequence tag no. 12052; clone no. 154J19T7), and PAL (PAL1, PAL2, and PAL3) (Wanner et al., 1995) were made using the DECAprime II system (Ambion, Austin, TX).

DNA Analysis and Sequencing

Genomic Arabidopsis DNA carrying the *F5H* coding sequence and 5′ and 3′ regulatory regions was subcloned into

pGEM-7Zf(+) (Promega) or pMBL18 (Nakano et al., 1995) for manual (Sequenase kit version 2.0 [United States Biochemical]) or automated (model 373A sequencer [Applied Biosystems] or model ALF Express sequencer [Pharmacia]) sequencing. The position of the T-DNA insertion in the *fah1-9* allele was determined by cloning and sequencing a 0.5-kb PCR product made by amplification of the junction between the T-DNA right border and the *F5H* genomic DNA using the PCR primers GCCAACCACGCGCCTCATCT (*F5H*) and GTCACCTTAGGCGACTTTTGA (T-DNA right border). The *F5H* transcription start site was determined by primer extension as described previously (Bell-Lelong et al., 1997) using the primer GAGACGTCGTGGGATCTGATAG.

Construction of Transgenic Lines

Standard techniques were used for DNA manipulations (Sambrook et al., 1989). The isolation of cosmid pBIC20-F5H, the construction of plasmids 35S-F5H and C4H-F5H, and the introduction of these three constructs into the *fah1-2* mutant line was described previously (Meyer et al., 1996, 1998). The F5H(HX) construct was made by ligating a 5.15-kb *Hin*dIII-*Xho*I fragment, derived from pBIC20-F5H and containing the *F5H* promotor and coding region, into the binary vector pGA482 (An, 1987). This plasmid was transformed into the *fah1-2* mutant by vacuum infiltration (Bent et al., 1994), as described previously (Bell-Lelong et al., 1997). Two representative lines containing a single T-DNA insertion were made homozygous and used for subsequent experiments.

Analysis of Sinapate Esters

Sinapate esters were extracted from plant tissues in 50% methanol containing 0.75% (v/v) phosphoric acid. A 20-μL sample of each extract was analyzed by HPLC on a C_{18} column (Microsorb-MV, Rainin Instruments, Woburn, MA) using a gradient from 1.5% phosphoric acid to 35% acetonitrile in 1.5% phosphoric acid for elution and UV detection at 335 nm. Sinapate esters were quantitated using the extinction coefficient of sinapic acid. For TLC analyses, seed sinapate esters were extracted in 50% methanol and separated on silica gel K6 TLC plates (Whatman) using a solvent mixture of *n*-butanol, acetic acid, and water (5:2:3, v/v), and visualized under UV light.

RESULTS

F5H Transcript Accumulation Is Distinct from That of Other Phenylpropanoid Pathway Genes

To evaluate the tissue specificity of *F5H* expression, the abundance of *F5H* transcript in various organs of wild-type Arabidopsis was examined (Fig. 2). RNA-blot analysis using the *F5H* cDNA (Meyer et al., 1996) as a probe indicated that *F5H* mRNA accumulated in all tissues examined. As a fraction of total RNA, the highest level of *F5H* message was found in the rachi, which is consistent with the role of F5H in lignin biosynthesis. *F5H* expression in mature leaves was substantially lower than that in older or younger leaves.

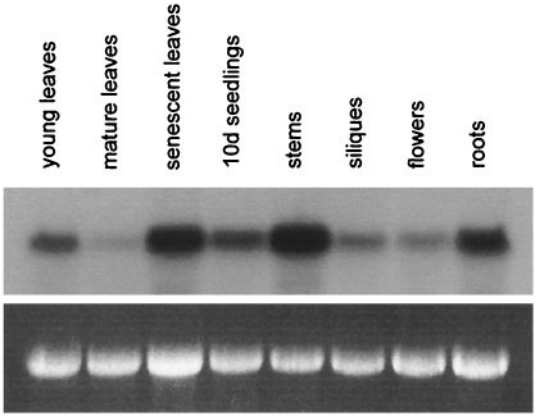

Figure 2. F5H transcript accumulation in tissues of wild-type Arabidopsis. Total RNA was extracted from the tissues indicated and probed with the *F5H* cDNA in RNA-blot analysis. The bottom panel illustrates ethidium-bromide staining of rRNA as a loading control.

To compare the expression of *F5H* with that of other phenylpropanoid genes, we determined their mRNA levels in developing light- and dark-grown seedlings and in seedlings grown in the dark for 4 d before transfer to the light. Except for a weak signal in 1-d-old seedlings, *F5H* transcript was nearly undetectable in dark-grown seedlings and in light-grown plants before d 3 (Fig. 3A). In light-grown seedlings *F5H* mRNA increased slowly over the remainder of the 10-d experimental period. When dark-grown seedlings were shifted to light conditions, *F5H* transcript levels increased during the next 6 d at approximately the same rate as in light-grown seedlings. This pattern of expression differed from that of the other genes examined. The mRNA of *PAL1*, *PAL2*, *C4H*, *OMT*, and *4CL* accumulated more rapidly than that of *F5H* in light-grown seedlings, reaching maximal levels within 4 d of planting (Fig. 3B). In etiolated seedlings mRNAs corresponding to all of these genes were readily detectable, but were generally lower than in light-grown plants. These transcripts still reached maximal levels in the dark within 4 d of planting, but then gradually decreased. In the shift experiments transcripts reached maximal levels within 2 d after transfer to the light. Consistent with a previous report (Wanner et al., 1995), *PAL3* mRNA accumulation was undetectable at all time points tested (data not shown).

Sinapoylmalate Accumulation in Seedlings Does Not Change in Response to Constitutive Expression of *F5H*

To correlate the results of the previous expression analysis with phenylpropanoid metabolism, the interconversion and biosynthesis of sinapate esters was evaluated in germinating light- and dark-grown wild-type seedlings (Fig. 4). In light-grown wild-type seedlings, the levels of sinapoylcholine decreased to undetectable levels by d 3. Coincident with this decrease in sinapoylcholine was a transient increase in sinapoylglucose, which peaked between d 2 and 3. By d 4 sinapoylglucose levels had decreased to nearly undetectable levels. Sinapoylmalate,

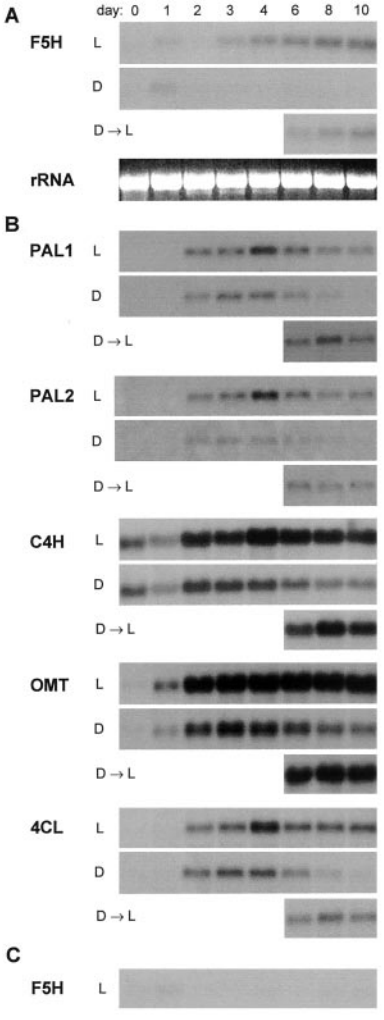

Figure 3. Transcript accumulation of phenylpropanoid genes during seedling development. Arabidopsis seedlings were germinated and grown under aseptic conditions on modified Murashige-Skoog agar plates (Lorenzen et al., 1996) in light (L; 16-h light/8-h dark photoperiod), in darkness (D), or in darkness for 4 d before being shifted to light conditions (D→L). Total RNA was extracted on the days indicated and RNA analysis was performed using probes from cDNAs of the indicated genes. A, Total RNA from wild-type seedlings probed with the *F5H* cDNA. The lower panel illustrates ethidium-bromide staining of rRNA as a loading control. B, Total RNA from wild-type seedlings probed with cDNAs corresponding to the phenylpropanoid genes indicated. C, Total RNA from a *fah1-2*: F5H(HX) transgenic line probed with the *F5H* cDNA. All blots were exposed to film for 24 h except PAL2, which was exposed for 48 h.

which is undetectable in seeds, began to accumulate by d 2 and increased until at d 6 it was the dominant sinapate ester. By this time its levels approximated the total sinapate ester content of seeds. Sinapoylmalate continued to increase in abundance each day thereafter, indicating that de novo synthesis begins to contribute to the sinapate ester content of wild-type seedlings at approximately d 6. In dark-grown wild-type seedlings, the levels of sinapoylcholine and sinapoylglucose were found to be nearly the same as in light-grown seedlings for the first 2 d after imbibition.

During the next 8 d the levels of sinapoylglucose and, to a lesser extent, sinapoylcholine remained elevated, and sinapoylmalate failed to accumulate. The total sinapate ester content of dark-grown seedlings never exceeded the levels found in seeds, suggesting that de novo synthesis was not initiated under these conditions. When dark-grown seedlings were transferred to the light on d 4, sinapoylglucose levels decreased and sinapoylmalate levels increased gradually, indicating that transfer to the light permitted the completion of the interconversion phase and the onset of the de novo phase of sinapate ester biosynthesis. In both light- and dark-grown seedlings transferred to the light, the onset of de novo sinapate ester biosynthesis coincided with the initiation of *F5H* mRNA accumulation, which is consistent with the hypothesis that *F5H* expression regulates the biosynthesis of these metabolites in developing Arabidopsis seedlings.

To test the hypothesis that de novo accumulation of sinapoylmalate in seedlings is determined at the level of *F5H* expression, sinapate ester content was compared in light- and dark-grown transgenic seedlings constitutively expressing *F5H* (Fig. 4). If *F5H* expression were rate limiting for de novo sinapoylmalate biosynthesis, it would be expected that its constitutive expression would cause a precocious accumulation of sinapoylmalate in light-grown seedlings and possibly permit its accumulation in etiolated seedlings. Instead, constitutive expression of *F5H* under the direction of the CaMV 35S promoter in the *fah1-2*:35S-F5H transgenic line (Fig. 5) had virtually no effect on the onset of sinapoylmalate biosynthesis (Fig. 4). This result argues that *F5H* expression is not a control point for the temporal regulation of sinapoylmalate accumulation. Furthermore, the absence of sinapoylmalate in dark-grown *fah1-2*:35S-F5H seedlings suggests that additional light-dependent factors are required for sinapoylmalate accumulation in etiolated seedlings.

F5H Expression Does Not Determine Sinapoylmalate Distribution in Mature Leaves

The distribution of sinapoylmalate in Arabidopsis leaves can be visualized by examining plants under UV light. Sinapoylmalate leads to a blue-green fluorescence in the adaxial epidermis, whereas the abaxial epidermis fluoresces red. The adaxial surfaces of rosette leaves of wild type, *fah1-2*, and the *fah1-2* transgenic lines are indistinguishable when observed under white light (Fig. 6). Under UV light sinapoylmalate in leaves of the wild-type and the *fah1-2*:35S-F5H and *fah1-2*:C4H-F5H transgenic plants (Fig. 5) causes them to appear blue-green. In contrast, the leaves of the *fah1* mutant appear red because they lack sinapoylmalate (Chapple et al., 1992). These data are consistent with previous biochemical analyses showing that the 35S-F5H and C4H-F5H constructs are capable of complementing the *fah1-2* mutant phenotype (Meyer et al., 1996, 1998). Under UV illumination the abaxial surfaces of the wild-type, mutant, and transgenic leaves appear red (Fig. 6), indicating the absence of sinapoylmalate. Thus, ectopic overexpression of *F5H* did not lead to the accumulation of sinapoylmalate in the abaxial leaf epidermis. Although *F5H*

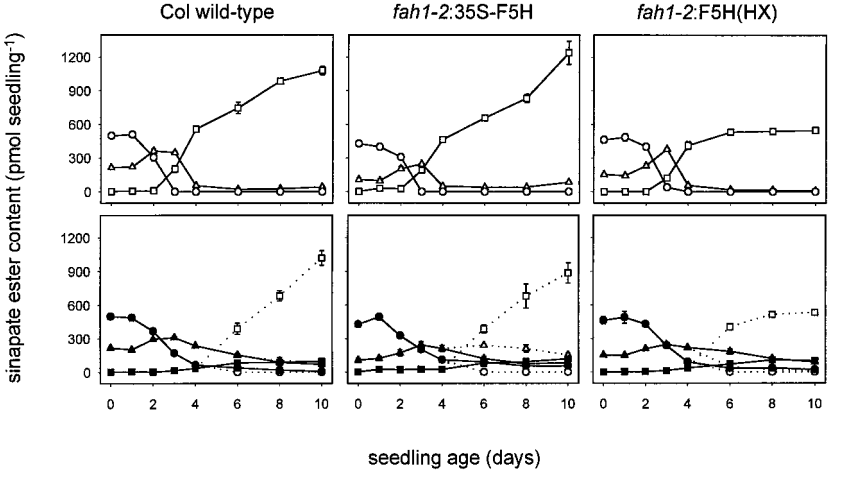

Figure 4. Accumulation of sinapate esters in wild-type and transgenic *fah1* Arabidopsis seedlings. Wild-type seedlings and transgenic *fah1-2* seedlings were grown on modified Murashige-Skoog agar plates as described for Figure 3. Sinapate esters were fractionated by HPLC, detected at 335 nm, and quantitated using the extinction coefficient of sinapic acid. Each point represents the average of three replicates of 10 seedlings each ± SE. Top row, Seedlings grown in the light (open symbols). Bottom row, Seedlings grown in the dark (solid lines, solid symbols) or shifted from dark to light on d 4 (dotted lines, open symbols). Circles, Sinapoylcholine; triangles, sinapoylglucose; squares, sinapoylmalate.

expression determines the spatial deposition of syringyl units in lignifying tissues (Meyer et al., 1998), this result demonstrates that ectopic *F5H* expression is not sufficient to lead to sinapoylmalate accumulation in the abaxial leaf epidermis.

Sinapoylcholine Accumulation in Embryos Is Not Regulated at the Level of F5H Transcription

We examined *F5H* transcript accumulation in developing siliques of the wild type and various transgenic lines to investigate whether *F5H* expression could be the control point for sinapoylcholine biosynthesis in developing embryos (Fig. 7). For the collection of these data total RNA was prepared from siliques of the primary inflorescence that had been sampled in pairs, beginning with the first expanding silique. *F5H* mRNA in transgenic *fah1-2* plants can only be the result of transgene expression, because the mutant lacks endogenous F5H transcript (Meyer et al., 1996). In the wild type *F5H* mRNA was nearly undetectable in the first five silique pairs and increased gradually thereafter. The pattern of *F5H* expression was similar in the *fah1-2*:pBIC20-F5H line. In contrast, a high level of *F5H* transcript was detected in the youngest siliques of both the *fah1-2*:35S-F5H and the *fah1-2*:C4H-F5H lines and this level remained relatively constant over the course of silique development. To determine whether the alteration of *F5H* expression had an effect on sinapate ester biosynthesis, the accumulation of sinapoylcholine was examined in comparable siliques (Fig. 8). As expected from earlier studies (Chapple et al., 1992), this compound was nearly undetectable in the *fah1-2* mutant. In the wild type, sinapoylcholine was first detected in the fifth to seventh silique pairs, and its accumulation paralleled the increase in *F5H* expression. Although these data were consistent with a regulatory role for *F5H* expression, overexpression of *F5H* had no effect on the developmental onset of sinapoylcholine accumulation (Fig. 8). It seems unlikely that transcriptional regulation of *F5H* is a control point for sinapoylcholine biosynthesis in embryos.

Sequences Downstream of the *F5H* Gene Are Required to Complement the *fah1* Mutant Phenotype

The pBIC20-F5H cosmid contains 2.5 kb of DNA upstream and approximately 12.5 kb of DNA downstream of the *F5H* coding region (Fig. 5) and complements both the sinapoylmalate and syringyl lignin deficiencies of the *fah1-2* mutant (Meyer et al., 1996). Similarly, transformation of the mutant with constructs in which expression of the *F5H* gene is driven by the CaMV 35S promoter (Odell et al., 1985) or the Arabidopsis C4H promoter (Bell-Lelong et al., 1997) restores all of the wild-type phenotypes (Meyer et

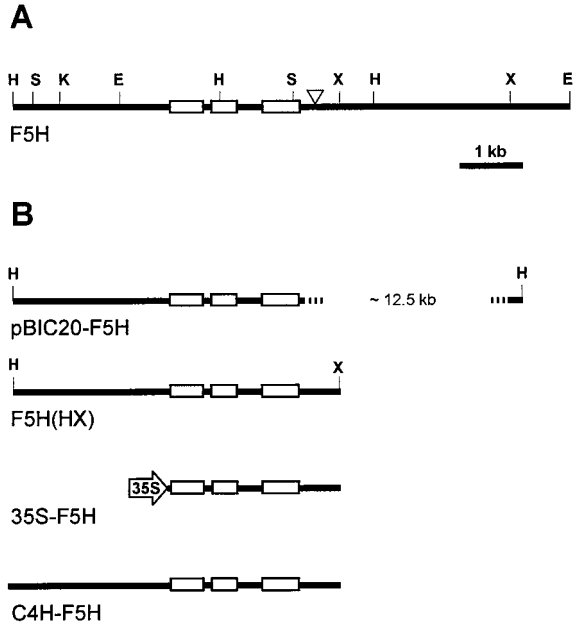

Figure 5. Diagrammatic representation of the *F5H* gene and *F5H* transgenes used in this study. Exons are represented by open rectangles. A, *F5H* genomic region. An inverted triangle indicates the location of a T-DNA insertion in the *fah1-9* mutant. B, Constructs used for *F5H* expression analysis in the *fah1-2* mutant background. 35S, CaMV 35S promoter; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sac*I; X, *Xho*I.

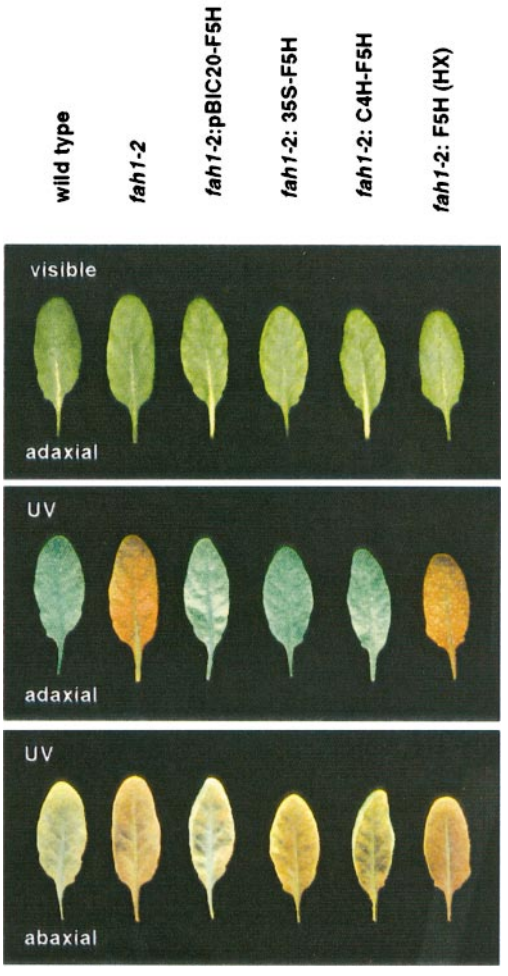

Figure 6. Leaves of wild type, *fah1-2*, and transgenic lines as viewed under visible and UV light. Top panel, Adaxial leaf surfaces illuminated by visible light; middle panel, adaxial leaf surfaces illuminated by UV light; bottom panel, abaxial leaf surfaces illuminated by UV light (peak wavelength = 302 nm).

al., 1996, 1998; this study). To further delimit the region of DNA sufficient to direct *F5H* gene expression, the plasmid F5H(HX) (Fig. 5) was introduced into *fah1-2* plants. Despite having the same upstream DNA as pBIC20-F5H and the same amount of downstream DNA as the 35S-F5H and C4H-F5H constructs, observation under UV light of more than 50 independent transgenic lines indicated that F5H(HX) failed to complement the *fah1-2* mutant phenotype (Fig. 6; data not shown). To explore this observation further, *F5H* mRNA abundance was examined in homozygous *fah1-2*:F5H(HX) transgenic seedlings. These analyses indicated that *F5H* transcript in *fah1-2*:F5H(HX) seedlings on d 1 (Fig. 3C) was similar to that in the wild type (Fig. 3A), but that there was no subsequent increase in *F5H* mRNA.

Although sinapoylmalate is absent in mature *fah1-2*:F5H(HX) plants, initial experiments demonstrated its presence in young seedlings (data not shown). To further explore these preliminary observations, the levels of sinapate esters were measured in developing *fah1-2*:F5H(HX) seedlings (Fig. 4). The sinapate ester profile of these seedlings was nearly identical to that of the wild type during the first 4 d of development. Thereafter, sinapoylmalate levels remained constant, which is consistent with the absence of *F5H* mRNA accumulation in seedlings of this transgenic line (Fig. 3C) and the requirement of *F5H* expression for de novo sinapoylmalate biosynthesis. *fah1-2*:F5H(HX) seeds (d 0) contained wild-type levels of sinapoylcholine. These data indicate that *F5H* must have been expressed during the development of the embryos within these seeds. As predicted by the presence of sinapoylcholine in *fah1-2*:F5H(HX) seeds (Fig. 4), developing siliques also accumulated sinapoylcholine (Fig. 8) and *F5H* transcript (Fig. 7). The high level of *F5H* expression in this line is likely the result of a transgene positional effect, because a second *fah1-2*:F5H(HX) line accumulated approximately wild-type levels of *F5H* transcript in maturing siliques (data not shown).

Our results indicate that the 630 bp of 3′ DNA in the 35S-F5H, C4H-F5H, and F5H(HX) constructs was sufficient for expression and mRNA stability in leaves when *F5H* was driven with a heterologous promoter, but in vegetative tissue *F5H* expression required additional downstream DNA in the context of its own promoter. From these data we conclude that an element in this downstream DNA functions as a positive regulator of gene expression. In contrast, the presence of F5H transcript (Fig. 7) and sinapoylcholine (Fig. 8) in *fah1-2*:F5H(HX) embryos indicates that the *F5H* 3′-flanking DNA is not required for *F5H* expression in developing embryos.

A T-DNA Insertion in the Downstream DNA of the *fah1-9* Allele Results in a Phenotype That Is Similar to That of the *fah1-2:F5H(HX)* Transgenic Line

To determine how the T-DNA insertion in the *fah1-9* allele leads to the mutant phenotype (Meyer et al., 1996), its

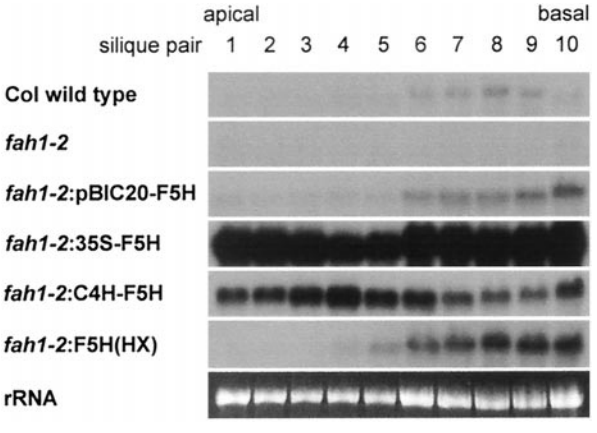

Figure 7. Accumulation of *F5H* transcript in developing siliques. Siliques were collected in pairs from 5-week-old plants of the lines indicated, beginning with the first expanding silique. Total RNA was extracted and probed with the *F5H* cDNA in RNA analysis. Blots were exposed to film for 24 h (*fah1-2*:35S-F5H and *fah1-2*:C4H-F5H) or 48 h. The bottom panel illustrates ethidium-bromide staining of rRNA as a loading control. Col, Arabidopsis ecotype Columbia.

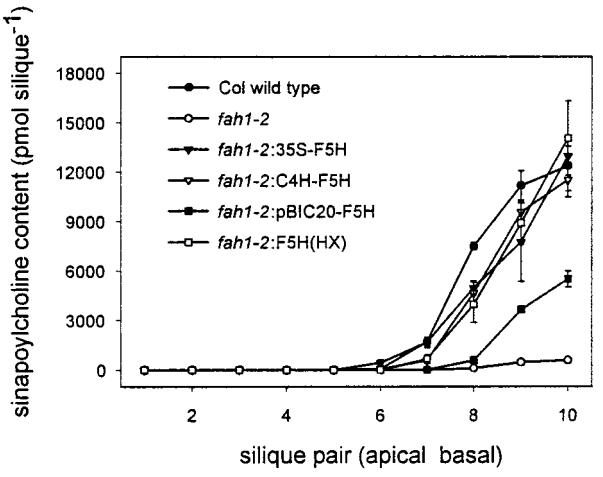

Figure 8. Accumulation of sinapoylcholine in developing siliques. Siliques were collected in pairs as described for Figure 7 and extracted in acidic 50% methanol. Sinapoylcholine was quantified by HPLC as described for Figure 4. Each point represents the average of three replicates of three silique pairs ± SE. Col, Arabidopsis ecotype Columbia.

position was determined by the cloning and sequencing of a PCR product made from the T-DNA right border-*F5H* junction. The T-DNA right border was found to be 283 bp 3′ of the *F5H* stop codon and 38 bp downstream of the *F5H* polyadenylation site (Fig. 5). This observation suggested that the T-DNA may interfere with the regulatory functions of the *F5H* 3′ DNA. Because the 3′-flanking DNA is not required for *F5H* expression in embryos, this hypothesis would predict that the *F5H* gene would be transcribed in *fah1-9* embryos and would permit sinapate ester accumulation. Indeed, sinapoylcholine is present at approximately wild-type levels in *fah1-9* seeds (Fig. 9). Together with the observation that *fah1-9* homozygotes fail to accumulate sinapoylmalate in leaves, these results provide independent genetic evidence for the regulatory role of the 3′ region of the *F5H* gene and its tissue-specific function.

DISCUSSION

Tracheophytes accumulate myriad phenylpropanoid derivatives that range from taxon-specific flavonoids and simple phenylpropanoid glucosides and esters to the more complex and ubiquitous phenylpropanoid polymer lignin. The sinapic acid esters sinapoylmalate and sinapoylcholine are most commonly found in members of the Brassicaceae. In Arabidopsis these compounds provide convenient endogenous fluorescent reporters of the activity of the phenylpropanoid pathway and the enzymes specific to sinapate ester biosynthesis. Using these markers in combination with conventional molecular approaches, we have demonstrated that *F5H* expression is modulated independently of other phenylpropanoid pathway genes in Arabidopsis, but does not regulate sinapate ester accumulation. These studies have also shown that *F5H* expression involves a regulatory element located 3′ of its stop codon, a feature not previously associated with phenylpropanoid pathway gene regulation.

Comparison of F5H Expression with That of Other Phenylpropanoid Genes

The core phenylpropanoid biosynthetic genes (*PAL*, *C4H*, and *4CL*) are expressed at relatively high basal levels even in dark-grown plants, and these transcripts increase in response to light and wounding (Hahlbrock and Scheel, 1989; Ohl et al., 1990; Logemann et al., 1995; Bell-Lelong et al., 1997; Mizutani et al., 1997). In contrast, the basal level of *F5H* expression in etiolated seedlings is very low compared with that of light-grown seedlings. In this respect *F5H* mRNA accumulation resembles that of the Arabidopsis flavonoid biosynthetic genes encoding chalcone synthase, chalcone isomerase, and dihydroflavonol reductase in developing dark-grown seedlings (Kubasek et al., 1992). The regulation of *F5H* is distinct, however, because in light-grown seedlings transcript levels for these three flavonoid genes peak at d 3 to 4 of growth and then decrease to nearly undetectable levels. The expression of *PAL*, *C4H*, *OMT*, and *4CL* shows a similar increase at d 4, although their transcripts persist at higher levels than those of the flavonoid genes during the next week of growth (Kubasek et al., 1992; this study). In contrast, *F5H* mRNA accumulates continuously for the first 10 d of seedling development.

In mature plants *F5H* mRNA was found in all organs examined, but was most abundant in the rachis, which is consistent with the role of F5H in the lignification of the sclerified interfascicular parenchyma. *F5H* transcript was also abundant in young leaves, where it is required for the synthesis of UV-protective sinapoylmalate during leaf expansion. The relatively low levels of *F5H* mRNA in mature leaves may suggest that the sinapoylmalate synthesized in

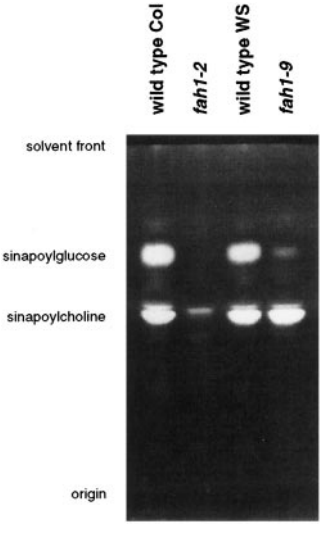

Figure 9. Sinapate ester accumulation in seeds of the *fah1-9* mutant. Seed extracts were separated by TLC and photographed under UV light. Col, Arabidopsis ecotype Columbia; WS, Arabidopsis ecotype Wassilewskija.

young leaves is relatively stable and can serve as a UV protectant during the lifetime of the leaf. Alternatively, it may indicate that the F5H protein has a long half-life and can support continued synthesis of sinapoylmalate.

The high levels of *F5H* mRNA in senescent leaves are more difficult to explain, because sinapoylmalate levels are known to decline in older rosettes (Chapple et al., 1992). High levels of transcript in these leaves may reflect continued synthesis of sinapoylmalate coupled with rapid turnover or fortuitous expression that is not correlated with secondary metabolite production caused by a limitation imposed by another step in the biosynthetic pathway. The high level of expression in roots is also surprising, because we have not been able to detect substantial levels of sinapate-derived metabolites in this tissue (M. Ruegger and C. Chapple, unpublished results). *C4H* is also highly expressed in Arabidopsis roots (Bell-Lelong et al., 1997), suggesting that phenylpropanoid gene expression is activated for the production of compounds that have not yet been identified, perhaps because of secretion or transport to the aerial portion of the plant. Alternatively, the levels of *F5H* expression in roots and senescent leaves may represent stress induction of phenylpropanoid gene expression. We believe that this is unlikely because, although many experiments have demonstrated the wound inducibility of the core phenylpropanoid genes (Ohl et al., 1990; Bell-Lelong et al., 1997; Mizutani et al., 1997), we have been unable to detect wound induction of *F5H* transcript (data not shown).

F5H Is Not a Regulatory Site for Sinapate Ester Biosynthesis

By using alternative promoters to overexpress *F5H*, we have demonstrated that its transcription regulates the flux of phenylpropanoid precursors through sinapic acid and toward syringyl lignin in lignifying cells of Arabidopsis (Meyer et al., 1998). The same study also showed that xylem-targeted expression of *F5H* was sufficient to permit the deposition of syringyl lignin in cells that normally accumulate only guaiacyl lignin. In contrast, although *F5H* expression parallels the accumulation of sinapate esters in developing seedlings and siliques, overexpression of *F5H* has no effect on the temporal regulation of their appearance. Similarly, although sinapoylmalate is distributed in a tissue-specific fashion, ectopic expression of *F5H* is not sufficient to permit its accumulation in the abaxial epidermis. It could be argued that, as in previous lignin-modification studies, *F5H* expression must be targeted to the specific cells in which sinapate esters are made and that our overexpression constructs failed to do so. On the other hand, we believe that sinapate ester synthesis is a cell-autonomous trait, and because C4H activity is required for sinapate ester synthesis, the *C4H* promoter should effectively target *F5H* expression to the correct cells. Thus, although *F5H* expression does control the biosynthesis of syringyl lignin, we conclude that it does not have a general regulatory role in the production of sinapic acid-derived metabolites.

These results leave open the question of how sinapate ester synthesis is regulated in Arabidopsis. It seems unlikely that sinapoylmalate synthesis is regulated by the activity of sinapoylglucose:malate sinapoyltransferase, because sinapoylglucose does not accumulate in wild-type leaves. Sinapoylglucose:choline sinapoyltransferase may be a regulatory point in sinapoylcholine synthesis, because low levels of sinapoylglucose are found in seeds of the Columbia ecotype of Arabidopsis. Free sinapic acid is found in neither seeds nor leaves, suggesting that sinapic acid:UDPG sinapoyltransferase does not have a regulatory role. Thus, steps earlier in the pathway probably regulate the synthesis of sinapate esters. The light independence and rapid accumulation of *PAL*, *C4H*, and *OMT* mRNAs in developing seedlings make it unlikely that sinapate ester synthesis is transcriptionally regulated within the phenylpropanoid pathway. The expression pattern of these genes is very different from the gradual and light-dependent increase in seedling sinapoylmalate content. The inability of *F5H* overexpression to alter sinapoylcholine accumulation in siliques leads to a similar conclusion.

These data suggest that if sinapate ester biosynthesis is controlled upstream of *F5H*, its regulation may be posttranscriptional in nature. Alternatively, transcriptional regulation may occur within the shikimate pathway, which supplies Phe for phenylpropanoid biosynthesis. Finally, with regard to the adaxial specificity of sinapoylmalate accumulation, the possibility that sinapate esters or their precursors are synthesized and then transported to the upper epidermis has not been excluded. Ectopic expression of *F5H* would not be expected to alter a transport or source/sink mechanism involved in such a movement of metabolites.

The Role of Downstream DNA in Gene Expression

The *fah1-9* mutant and the *fah1-2*:F5H(HX) transgenic lines failed to accumulate sinapoylmalate in their leaves. In contrast, these same lines accumulated *F5H* transcript and sinapoylcholine in developing siliques and seeds, indicating that the 3′-flanking DNA necessary for *F5H* expression in adult tissues is not required for expression in embryos. A number of reports have demonstrated the involvement of 3′-flanking DNA in plant gene expression (Thornburg et al., 1987; Dean et al., 1989; Elliott et al., 1989; Dietrich et al., 1992; Larkin et al., 1993; Viret et al., 1994; Fu et al., 1995a, 1995b; Chinn et al., 1996; Marshall et al., 1997). Downstream sequences have been shown to be required for gene expression in response to wounding (Thornburg et al., 1987), light (Viret et al., 1994), and Suc (Fu et al., 1995a). They have also been shown to be necessary for correct spatial expression either by activating (Dietrich et al., 1992; Larkin et al., 1993) or repressing (Viret et al., 1994) gene expression. Certain 3′-flanking sequences are thought to act at the level of transcriptional regulation (Dean et al., 1989; Larkin et al., 1993; Viret et al., 1994), whereas others are thought to affect mRNA stability (Elliott et al., 1989) or chromatin structure (Chinn et al., 1996).

The requirement for 3′-flanking sequences in *F5H* expression further differentiates the regulation of *F5H* from

that of other phenylpropanoid genes. Thus, the regulatory factors that control *F5H* expression in Arabidopsis may be independent of those that control upstream genes. This observation may be related to the fact that sinapate ester accumulation is taxonomically restricted, and that sinapate-derived secondary metabolites are less common than derivatives of hydroxycinnamic acids such as caffeic and ferulic acids. A recombination or transposition event that positioned a regulatory element downstream of the *F5H*-coding sequence early in the evolutionary history of the Brassicaceae may have been critical to the acquisition of the ability to express *F5H* and to accumulate sinapic acid derivatives in leaf tissue.

The differential requirements for *F5H* expression in seedlings and leaves versus embryos indicate that the 3′-flanking DNA may be a determining factor for tissue specificity. Although the mechanism underlying this activity remains to be characterized, our preliminary results suggest that it is unlikely to involve mRNA stabilization. The 35S-F5H and C4H-F5H constructs, both of which lack the 3′ DNA, lead to high levels of *F5H* mRNA in transformed plants (Meyer et al., 1996, 1998). These data suggest that the 3′ region is not required for *F5H* transcript stability. On the other hand, these relatively strong promoters may compensate for the absence of the stabilization provided by the 3′ sequence, which might be important in the context of the relatively weak *F5H* promoter. Preliminary results indicate that the 3′ DNA is required for expression of an *F5H* promoter-driven *GUS* reporter gene in leaves and stems of adult plants, but is not required for expression in embryos (M. Ruegger and C. Chapple, unpublished results). These results also argue against a role for the 3′ sequences in transcript stability. By the addition of downstream restriction fragments to the F5H(HX) construct and complementation of the *fah1* leaf phenotype, we have recently shown that sequences that constitute this downstream regulatory element are contained within a 3326-bp region 3′ of the F5H stop codon (M. Ruegger and C. Chapple, unpublished results).

The Utility of Sinapate Ester Accumulation as a Tool to Understand Plant Gene Regulation

The ease of detection and dispensable nature of sinapate esters make Arabidopsis an attractive model for the study of phenylpropanoid gene regulation. Although the requirement for 3′-flanking sequences has been demonstrated for a number of plant genes, we are unaware of any reports in which downstream *cis*-acting elements have been defined in detail or shown to be involved in phenylpropanoid gene expression. We believe that the study of secondary metabolism in Arabidopsis, particularly with respect to *F5H* expression and sinapate ester accumulation, can provide critical insights into these and other factors that regulate gene expression. The use of *F5H*-containing transgenes for mutant complementation avoids issues associated with most reporter-gene experiments, such as stability of the transgene product and the elimination or altered spatial organization of *cis*-acting elements. Unlike many reporter constructs, *F5H* transgenes contain a full complement of *cis*-acting elements in their native context. When introduced into the *fah1* mutant, indirect but quantitative assays of F5H activity and expression can be made by the determination of sinapate ester content in embryos and leaves, and syringyl lignin content in stems. Finally, the isolation of novel sinapate ester-deficient mutants may lead to the identification of *trans*-acting factors that regulate *F5H* and/or other phenylpropanoid biosynthetic genes.

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