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Mutations That Reduce Sinapoylmalate Accumulation in *Arabidopsis thaliana* Define Loci With Diverse Roles in Phenylpropanoid Metabolism

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ABSTRACT

The products of phenylpropanoid metabolism in Arabidopsis include the three fluorescent sinapate esters sinapoylglucose, sinapoylmalate, and sinapoylcholine. The sinapoylmalate that accumulates in cotyledons and leaves causes these organs to appear blue-green under ultraviolet (UV) illumination. To find novel genes acting in phenylpropanoid metabolism, Arabidopsis seedlings were screened under UV for altered fluorescence phenotypes caused by changes in sinapoylmalate content. This screen identified recessive mutations at four *Reduced Epidermal Fluorescence* (*REF*) loci that reduced leaf sinapoylmalate content. Further analyses showed that the *ref* mutations affected other aspects of phenylpropanoid metabolism and some led to perturbations in normal plant development. A second class of mutations at the *Bright Trichomes 1* (*BRT1*) locus leads to modest reductions in sinapate ester content; however, the most notable phenotype of *brt1* mutants is the development of hyperfluorescent trichomes that appear to contain elevated levels of sinapate esters when compared to the wild type. These results indicate that at least five new loci affecting the developmentally regulated accumulation of phenylpropanoid secondary metabolites in Arabidopsis, and the cell specificity of their distribution, have been identified by screening for altered UV fluorescence phenotypes.

THE products of the phenylpropanoid pathway have a wide array of important functions in plants. Soluble products of the pathway include pigments, important UV protectants, and phytoalexins, as well as signaling molecules involved in plant pathogen interactions. The most notable insoluble pathway product is lignin, a polymeric phenolic compound that is deposited in the plant secondary cell wall to provide rigidity and decay resistance to sclerified tissues such as xylem.

The study of plants altered in the accumulation of pigmented phenylpropanoid end products has a long history in plant biology and has provided important insights into a wide range of phenomena. Studies of maize flavonoid mutants led to the discovery of transposable elements (reviewed in Freeling 1984) as well as the identification of genes, later found to encode transcription factors, which regulate the phenylpropanoid pathway (Dooner *et al.* 1991). Other work using maize and petunia mutants has demonstrated an important role for flavonoids in pollen viability (Mo *et al.* 1992; van der Meer *et al.* 1992). Finally, early studies in the modification of gene expression using sense suppression technologies relied on the colored anthocyanin pigments of petunia flowers as reporter molecules (Napoli *et al.* 1990; van der Krol *et al.* 1990).

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Like the maize flavonoid mutants, another group of phenylpropanoid pathway mutants manifests a visible phenotype that facilitated their identification. These include the maize *bm* and the sorghum *bmr* (*brown midrib*) mutants (Cherney *et al.* 1991; Vignols *et al.* 1995; Halpin *et al.* 1998). Defects in these genes lead to alterations in lignin chemistry that result in the deposition of a pigmented lignin polymer, which is readily observable in the sclerified bundle sheath extensions of the midrib. Related phenotypes have been recapitulated in tobacco and poplar using antisense and cosuppression to downregulate expression of lignin biosynthetic enzymes (Halpin *et al.* 1994; Atanassova *et al.* 1995; Van Doorsselaere *et al.* 1995; Baucher *et al.* 1996; Piquemal *et al.* 1998). Both these transgenic plants and the *brown midrib* mutants have attracted substantial interest because of their potential to improve the utilization efficiency of lignocellulosic materials in agriculture and industry.

In contrast to the large number of mutations that affect the accumulation of pigmented phenylpropanoids, few mutations are known that affect the biosynthesis of soluble hydroxycinnamic acid-derived secondary metabolites. Despite their potential as genetic markers for further dissection of the phenylpropanoid pathway, it is likely that the colorless nature of hydroxycinnamic acid-derived secondary metabolites has made this class of mutants a less obvious target for research.

Arabidopsis and other members of the Brassicaceae accumulate hydroxycinnamic acid esters that are fluorescent when exposed to UV light. These compounds

include a leaf-specific ester, sinapoylmalate, a seed-specific ester, sinapoylcholine, and their common biosynthetic precursor, sinapoylglucose. In leaves and seeds, sinapoylmalate and sinapoylcholine are synthesized *de novo* via the phenylpropanoid pathway. In contrast, the sinapoylmalate found in seedling cotyledons is derived from seed reserves of sinapoylcholine via a series of hydrolysis, conjugation, and transesterification reactions that have been well described at the biochemical and genetic levels (Lorenzen *et al.* 1996). Mutants that are defective in leaf sinapate ester biosynthesis can readily be identified since the fluorescent nature of these compounds can be visualized *in vivo* (Chapple *et al.* 1992). Mutations that lead to qualitative or quantitative changes in sinapate ester content in Arabidopsis either decrease this fluorescence (Lehfeldt *et al.* 2000) or reveal the chlorophyll fluorescence that makes the sinapate ester-deficient *fah1* mutant appear red under UV light (Ruegger *et al.* 1999). Although the *fah1* mutant was identified originally by thin layer chromatography of methanolic leaf extracts, the mutant's *in vivo* fluorescence phenotype was subsequently used to identify a T-DNA tagged allele that was used to isolate the *FAH1* gene (Meyer *et al.* 1996). Since the original *fah1* mutant screen was semiquantitative, it permitted the isolation of mutants with very strong phenotypes only. Thus, we suspected that a number of mutants with phenotypes more subtle than that exhibited by *fah1* might have so far escaped detection. To determine whether this strategy could be used to identify other loci required for phenylpropanoid metabolism in Arabidopsis, we conducted a screen to isolate novel mutants with altered leaf fluorescence phenotypes.

MATERIALS AND METHODS

Plant material and growth conditions: All Arabidopsis lines used for these experiments were of the Columbia ecotype except for *ref3-1*, which was isolated from the Landsberg erecta (Ler) ecotype, and *ref1-3* (Wassilewskija ecotype), which was isolated from a screen of 65,000 T-DNA mutagenized lines. Plants were grown under a 16 hr light/8 hr dark photoperiod in ProMix potting mix (Premier Horticulture, Red Hill, PA) at 22° in a growth chamber (Percival Scientific, Boone, IA).

Identification of mutants: M_2 seed from 237 seed pools, each pool derived from ~200 ethyl methanesulfonate-mutagenized M_1 plants, was sown to soil to give ~100,000 M_2 seedlings. Seven days after planting, all of the seedlings were examined under a transilluminator with a peak wavelength of 302 nm (model TM-36; UVP, San Gabriel, CA) in a dark room. The seedlings were rescreened 10–14 days later to identify additional mutants whose phenotypes were not obvious in the initial screen. Seedlings having a mutant phenotype were transferred to separate pots for further growth and observation. In subsequent analyses, a hand-held transilluminator (model ENF-2406; Spectronics, Westbury, NY) with a peak wavelength of 365 nm, was used as a source of UV. All lines used in subsequent experiments were backcrossed at least two times to wild type.

Photography: Plant specimens were placed inside of an aluminum foil-lined box and illuminated with a transilluminator (UVP TM-36). A yellow filter (yellow 2, no. 8; Tiffen, Hauppage, NY) was used to color correct images that were recorded on Ektachrome 400 film (Eastman Kodak, Rochester, NY). Further color correction was made using Photoshop (Adobe, San Jose, CA).

Analysis of sinapate esters: Sinapate esters were extracted from plant tissues in 50% methanol containing 0.75% (v/v) phosphoric acid (solvent A). For leaf analysis, single 3-week-old plants were extracted in 1 ml of solvent A per 100 mg of tissue at 65° for 1 hr. For seed analysis, 10 seeds were ground in 1 ml of solvent A in a 1.5-ml tube using a plastic pestle. A 20-μl sample of each extract was analyzed by HPLC on a C_{18} column (Microsorb-MV; Ranin 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 mm. Sinapate esters were quantified using the extinction coefficient for sinapic acid.

Lignin analysis: Cell wall material was prepared from stems of Arabidopsis plants as described previously (Meyer *et al.* 1998) with minor modifications. Briefly, stems were harvested from 8-week-old plants, dried under vacuum, and then ground in 0.1 M phosphate buffer, pH 7.2. The ground material was extracted three times with 70% ethanol at 80°, once with acetone, and then dried in air. Total lignin was assayed by thioglycolic acid derivatization (Campbell and Ellis 1992). Lignin monomer composition was determined by alkaline nitrobenzene oxidation (Iiyama and Lam 1990), modified as described previously (Meyer *et al.* 1998).

RESULTS

Mutants with altered leaf fluorescence can be identified under UV light: To identify mutants defective in sinapoylmalate biosynthesis, ~100,000 M_2 seedlings (Columbia ecotype) were screened at 1 week of age for seedlings that exhibited altered cotyledon fluorescence. Since sinapoylmalate accumulation in cotyledons is dependent upon seed reserves of sinapoylcholine, mutants of this type might be defective in sinapoylcholine biosynthesis or genes required for the interconversion of sinapoylcholine to sinapoylmalate. After embryonic stores of sinapate esters were depleted 3–7 days later (Lorenzen *et al.* 1996), the seedlings were rescreened to identify additional lines that failed to synthesize sinapoylmalate *de novo* in their developing leaves. Potential mutants were transplanted to separate pots and allowed to self-fertilize. Lines that displayed an altered fluorescence in the M_3 generation were backcrossed to wild type and, depending on their UV phenotype, were tested for complementation with known mutants affected in sinapate ester metabolism. These mutants included the red-fluorescent, sinapate ester-deficient *fah1-2* mutant (Chapple *et al.* 1992) and *sng1*, which accumulates sinapoylglucose instead of sinapoylmalate in its cotyledons and displays a dull-green UV fluorescence phenotype (Lehfeldt *et al.* 2000). In addition to the above crosses, pairwise complementation crosses were made among lines that complemented *fah1* and *sng1* and showed novel fluorescent or developmental phenotypes. Using this approach, we identified 34 new *fah1* alleles and 5 new *sng1* alleles (data not shown), as well as 21 indepen-

TABLE 1

Complementation analyses of Arabidopsis *ref* and *brt* mutants

Cross	Total	UV phenotype[a]	
		Wild type	Mutant
ref1-1/ref1-1 × *ref1-2/ref1-2*	25	0	25
ref1-2/ref1-2 × *ref1-3/ref1-3*[b]	17	0	17
ref1-1/ref1-1 × *ref1-4/ref1-4*	26	0	26
ref1-1/ref1-1 × *ref1-5/ref1-5*	37	0	37
ref1-1/ref1-1 × *ref1-6/ref1-6*	24	0	24
ref1-1/ref1-1 × *ref1-7/ref1-7*	28	0	28
ref1-2/ref1-2 × *ref2-1/ref2-1*	34	34	0
ref3-2/ref3-2 × *ref1-2/ref1-2*	18	18	0
ref4-1/ref4-1 × *ref1-2/ref1-2*	16	16	0
brt1-1/brt1-1 × *ref1-1/ref1-1*	22	22	0
ref2-1/ref2-1 × *ref2-2/ref2-2*[c]	37	0	37
ref2-1/ref2-1 × *ref2-3/ref2-3*[c]	16	0	16
ref2-1/ref2-1 × *ref2-4/ref2-4*[c]	16	0	16
ref3-2/ref3-2 × *ref2-1/ref2-1*	24	24	0
ref4-1/ref4-1 × *ref2-1/ref2-1*	37	37	0
brt1-1/brt1-1 × *ref2-1/ref2-1*	26	26	0
ref3-2/ref3-2 × *ref3-1/ref3-1*[d]	15	0	15
ref3-2/ref3-2 × *ref3-3/ref3-3*	32	0	32
ref3-3/ref3-3 × *ref3-4/REF3*[e]	50	30	20
ref3-2/ref3-2 × *ref4-1/ref4-1*	8	8	0
ref4-1/ref4-1 × *ref4-2/ref4-2*	23	0	23
ref4-1/ref4-1 × *ref4-3/ref4-3*	22	0	22
brt1-1/brt1-1 × *ref4-1/ref4-1*	16	16	0
brt1-1/brt1-1 × *brt1-2/brt1-2*	24	0	24
brt1-1/brt1-1 × *brt1-3/brt1-3*	39	0	39
brt1-1/brt1-1 × *brt1-4/brt1-4*	19	0	19
brt1-1/brt1-1 × *brt1-5/brt1-5*	14	0	14
brt1-1/brt1-1 × *brt1-6/brt1-6*	20	0	20
brt1-4/brt1-4 × *brt1-7/brt1-7*	21	0	21
brt1-4/brt1-4 × *brt1-8/brt1-8*	18	0	18

[a] F_1 plants were scored under UV, 7–14 days after planting.
[b] *ref1-3* (WS ecotype) was isolated from a screen of 65,000 T-DNA mutagenized lines.
[c] *ref2-2, ref2-3*, and *ref2-4* (Columbia ecotype) were isolated in separate screens of EMS-mutagenized seeds (J. Humphreys and C. Chapple, unpublished results).
[d] *ref3-1* (Ler ecotype) was isolated separately from EMS-mutagenized seed (C. Chapple and C. R. Somerville, unpublished results).
[e] *ref3-4/ref3-4* is sterile. The chi-square value for an expected wild type:mutant ratio of 1:1 is 2.00 ($P > 0.05$).

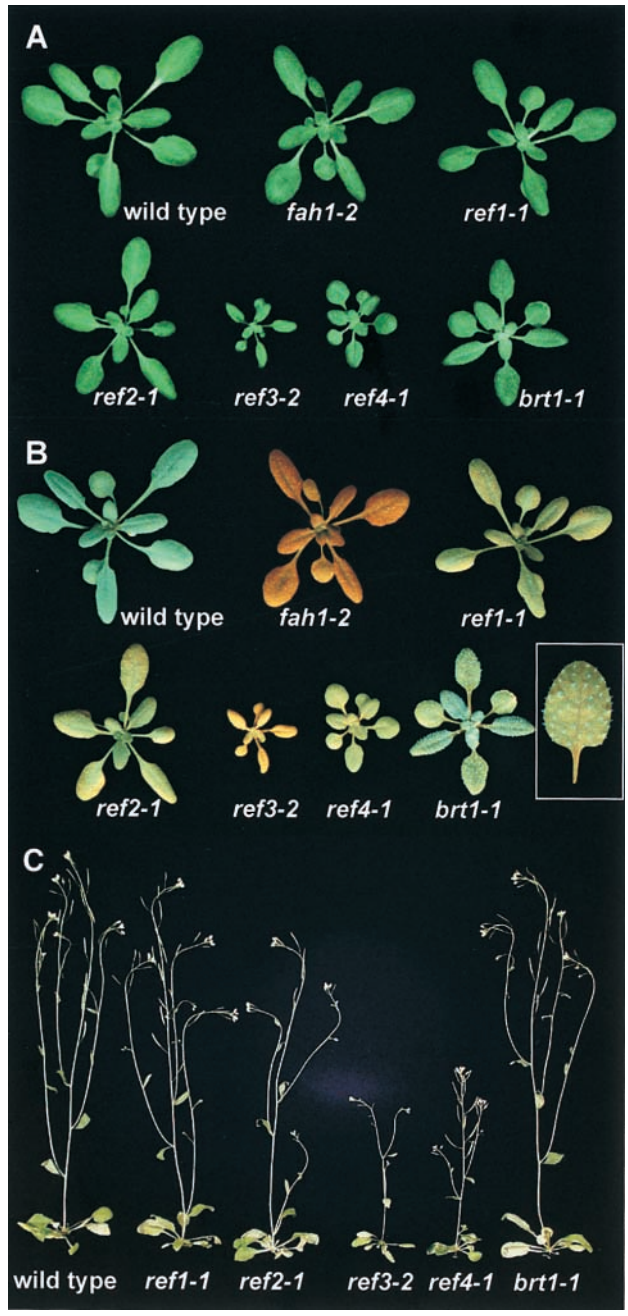

Figure 1.—Phenotypes of altered fluorescence mutants photographed under visible light (A and C) or UV (B). (A and B) Rosette-stage plants, 3 weeks after planting. The inset in B shows the hyperfluorescent trichome phenotype of the *brt1* mutant in greater detail. (C) Adult plants, 5 weeks after planting. The genotype of each plant is noted below.

dent mutants representing five novel loci that exhibit altered cotyledon and/or leaf fluorescence phenotypes (Table 1). Five additional mutant alleles at three of the loci were identified in similar, separate screens that had previously been conducted in efforts to isolate new *fah1* alleles (Table 1). Representatives of one class of mutants, *reduced epidermal fluorescence* (*ref*), display reductions in the blue-green fluorescence of their cotyledons and/or leaves (Figure 1), suggesting that these mutants accumulate lower levels of sinapoylmalate than the wild type. All of the *ref* mutants display a UV phenotype that is intermediate between wild type and the null *fah1-2* mutant. The leaves of a second class of mutants, *bright trichomes* (*brt*), are also less fluorescent than the wild type, but have trichomes that are hyperfluorescent under UV (Figure 1 inset). Since all of the *brt1* alleles were identified by the red-fluorescent UV phenotype of their cotyledons that lack trichomes, it is notable that the most obvious phenotype of *brt1* leaves was not used for the initial identification of most of these mutants. Although the metabolites responsible for the fluores-

TABLE 2

Genetic segregation of altered fluorescence mutations

Cross	Generation[b]	Total	UV phenotype[a]		χ^{2c}
			Wild type	Mutant	
REF1/REF1 × *ref1-1/ref1-1*	F_1	23	23	0	
	F_2	296	226	70	0.2883; $P > 0.05$
REF1/REF1 × *ref1-2/ref1-2*	F_1	24	24	0	
	F_2	323	231	92	2.0898; $P > 0.05$
REF2/REF2 × *ref2-1/ref2-1*	F_1	14	14	0	
	F_2	295	214	81	0.9503; $P > 0.05$
REF2/REF2 × *ref2-2/ref2-2*	F_1	10	10	0	
	F_2	239	179	60	0.0014; $P > 0.05$
REF3/REF3 × *ref3-2/ref3-2*	F_1	13	13	0	
	F_2	504	381	123	0.0952; $P > 0.05$
REF3/REF3 × *ref3-3/ref3-3*	F_1	27	27	0	
	F_2	234	175	59	0.0057; $P > 0.05$
REF4/REF4 × *ref4-1/ref4-1*	F_1	20	20	0	
	F_2	206	161	45	1.0939; $P > 0.05$
REF4/REF4 × *ref4-3/ref4-3*	F_1	23	23	0	
	F_2	184	140	44	0.1159; $P > 0.05$
BRT1/BRT1 × *brt1-1/brt1-1*	F_1	28	28	0	
	F_2	319	239	80	0.0010; $P > 0.05$
BRT1/BRT1 × *brt1-4/brt1-4*	F_1	24	24	0	
	F_2	262	197	65	0.0051; $P > 0.05$

[a] Plants were scored under UV, 7–14 days after planting.
[b] The F_2 generation represents the progeny from the self cross of the F_1 generation.
[c] Chi-square value for an expected wild type:mutant ratio of 3:1.

cence of *brt1* trichomes have not been identified, in a double mutant the *fah1* phenotype is epistatic to *brt1* (data not shown), strongly suggesting that the compounds are sinapate esters or related phenylpropanoids. When F_2 seedlings from crosses of all *ref* and *brt* mutants to wild type were examined, all of the mutant phenotypes segregated as recessive, nuclear, single gene mutations (Table 2).

***ref* and *brt* mutants are defective in sinapate ester biosynthesis:** Since mutations affecting the integrity of the photosynthetic apparatus or the tissue distribution of sinapate esters could lead to a *ref* phenotype, we analyzed extracts of 3-week-old rosettes by HPLC to directly quantify the impact of the *ref* and *brt* mutations on sinapoylmalate accumulation (Figure 2). As expected, sinapoylmalate was the major sinapate ester in wild-type Arabidopsis leaves (Chapple *et al.* 1992). Consistent with their visual phenotypes when observed under UV light, all of the *ref* mutants contained significantly less sinapoylmalate than the wild type. The *ref3* and *ref4* mutations form allelic series with respect to sinapoylmalate content, with *ref3-3* and *ref4-1* being the weakest alleles and *ref3-2* and *ref4-3* being the strongest alleles at their respective loci. Compared to the *ref* mutations, the *brt1* mutations lead to more modest reductions in sinapoylmalate content.

To determine whether the roles of the *REF* and *BRT* genes are restricted to sinapoylmalate production in leaves, or whether they have more general functions in hydroxycinnamic acid ester metabolism, we analyzed mutant seed extracts for sinapoylcholine content by HPLC (Figure 2). These analyses indicated that *ref1* and *ref3* mutations lead to reductions in seed sinapate ester levels that parallel the reductions in leaf sinapoylmalate content caused by these mutations (Figure 2). In contrast, *ref2*, *ref4*, and *brt1* mutations had little or no effect on seed sinapoylcholine accumulation. Surprisingly, *ref2* seed extracts contained a compound with a similar HPLC retention time and absorbance spectrum to that of sinapoylmalate, although the identity of this compound has not been unambiguously determined. These data indicate that *REF1* and *REF3* act in a portion of the phenylpropanoid pathway that is common to leaf and seed ester synthesis, whereas *REF2*, *REF4*, and *BRT1* are not required for sinapoylcholine biosynthesis or are functionally redundant with other genes that are expressed in developing embryos.

Lignin quantity and quality is affected in certain *ref* mutants: The sinapate ester phenotypes of the *ref* and *brt* mutations are consistent with defects in enzymes or regulatory factors required for the activity of the phenylpropanoid pathway. Quantitatively, lignin is one of the most significant products of this pathway, and we have previously shown that mutations affecting sinapate ester biosynthesis can also impact lignin biosynthesis (Chapple *et al.* 1992). Consequently, we examined the

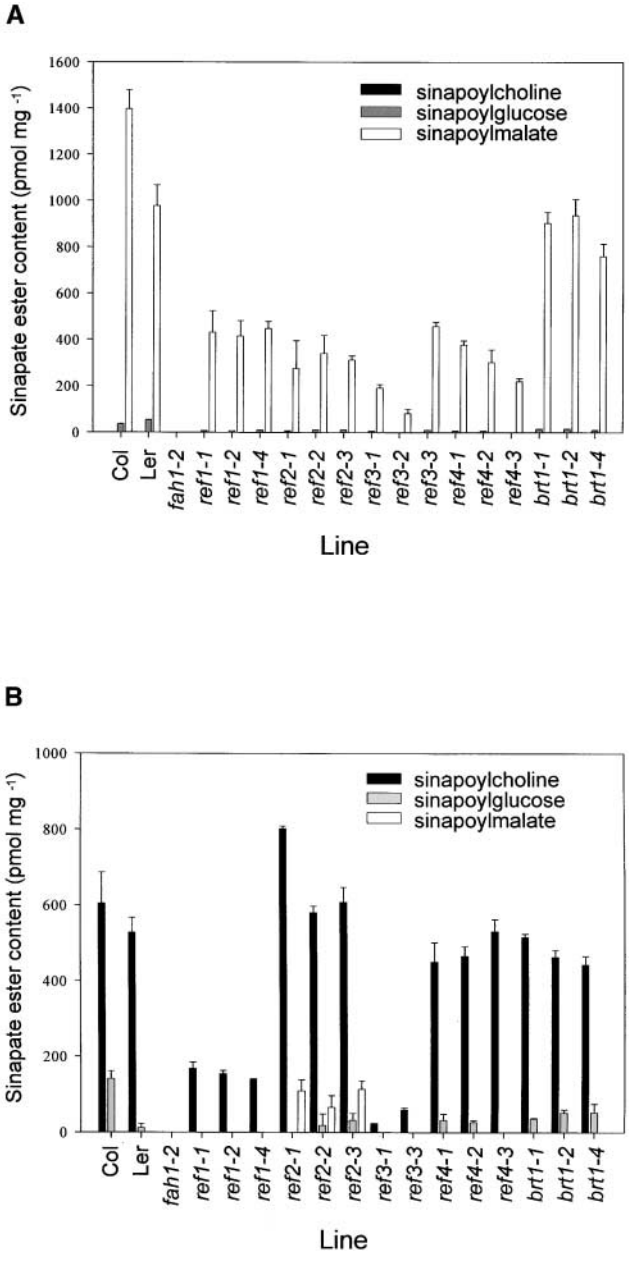

FIGURE 2.—Sinapate ester content of leaves and seeds of altered fluorescent mutants. Leaf and seed extracts were analyzed by HPLC and the amount of each of the three sinapate esters was determined using the extinction coefficient of sinapic acid. (A) Sinapate ester content of leaves. Each value represents the average sinapate ester content of five 3-week-old rosette plants. (B) Sinapate ester content of seeds. Each value represents the average sinapate ester content of three replicates, each replicate containing 10 seeds. The compound designated as sinapoylmalate in the *ref2* seed extracts exhibits the same HPLC retention time and UV spectrum; however, its identity has not been unambiguously demonstrated. Error bars represent one standard deviation.

impact of the *ref* and *brt* mutations on total lignin content, as well as lignin monomer composition. To measure the overall levels of the lignin polymer deposited in mutant stems, cell wall material from each of the lines was analyzed by thioglycolic acid (TGA) derivativization (CAMPBELL and ELLIS 1992). The results of these analyses indicate that *ref3* and *ref4* mutations lead to a significant decrease in stem lignin content, whereas the other mutations had little effect (Table 3).

TABLE 3

Lignin content of wild type and altered fluorescence mutants as determined by thioglycolic acid derivatization

Line	Lignin content (A_{280} mg^{-1} cell walls)
Wild type	1.67 ± 0.12
ref1-1	1.41 ± 0.10
ref1-2	1.46 ± 0.06
ref2-1	1.58 ± 0.06
ref2-2	1.81 ± 0.11
ref3-2	0.37 ± 0.12
ref3-3	0.68 ± 0.04
ref4-1	0.96 ± 0.10
ref4-3	0.70 ± 0.08
brt1-1	1.50 ± 0.30
brt1-4	1.39 ± 0.04

Lignin is synthesized via a branched pathway that leads to guaiacyl and syringyl substituted monomers, and only the latter class of subunits shares the ring substitution pattern common to the sinapate esters. To determine if any of the *ref* and *brt* mutations have an impact on only specific portions of the lignin biosynthetic pathway and thereby cause an alteration in lignin monomer composition, we analyzed the products from alkaline nitrobenzene oxidation (NBO) of saponified cell wall material by gas chromatography (IIYAMA and LAM 1990). Most of the mutations resulted in little or no change to lignin monomer composition (Table 4). The notable exceptions were the *ref2* mutants, in which a significant reduction in the lignin syringyl monomer content was observed. This result, coupled with the previous finding that *ref2* stems contain near wild-type levels of total lignin (Table 3), suggests that the *REF2* gene is required for the expression or activity of enzymes that direct phenylpropanoid pathway intermediates away from guaiacyl lignin biosynthesis and toward syringyl lignin deposition.

Flavonoid biosynthesis is perturbed in *ref3* and *ref4*: The seeds of *brt1* and most of the *ref* mutants are brown, consistent with the deposition of wild-type normal levels of phenylpropanoid pathway-derived condensed tannins (Figure 3). In contrast, the seed coats of *ref3* and *ref4* are less pigmented than wild type, although they are not as yellow as the chalcone synthase-deficient *tt4* mutant (BURBULIS *et al.* 1996). Similarly, stems of *ref3* and *ref4* plants display virtually none of the purple anthocyanin pigmentation that is characteristically seen at the basal region of wild-type stems (data not shown). These data suggest that the *REF3* and *REF4* genes play

TABLE 4

Lignin monomer composition in wild type and altered fluorescence mutants

Line	Total *G* units[a] (μmol g^{-1} d.w.)	Total *S* units[b] (μmol g^{-1} d.w.)	Total *G* + *S* units (μmol g^{-1} d.w.)	*S* (mol%)
Wild type	265 ± 31	131 ± 11	396 ± 41	33.0 ± 1.3
fah1-2	335 ± 64	ND	335 ± 64	—
ref1-1	320 ± 135	196 ± 56	516 ± 190	38.8 ± 3.7
ref1-2	406 ± 68	264 ± 90	670 ± 157	38.8 ± 3.9
ref2-1	210 ± 56	42 ± 16	252 ± 42	17.6 ± 8.6
ref2-2	354 ± 47	67 ± 18	421 ± 64	15.7 ± 1.8
ref3-2	63 ± 17	38 ± 12	101 ± 28	37.4 ± 1.3
ref3-3	122 ± 60	110 ± 68	232 ± 127	45.0 ± 6.4
ref4-1	168 ± 36	72 ± 16	240 ± 51	29.7 ± 1.4
ref4-3	154 ± 19	76 ± 17	230 ± 29	33.0 ± 4.6
brt1-1	303 ± 17	142 ± 6	446 ± 23	31.9 ± 0.7
brt1-4	256 ± 6	127 ± 6	383 ± 9	33.1 ± 1.0

ND, not detectable; d.w., dry weight.
[a] Sum of vanillin plus vanillic acid.
[b] Sum of syringaldehyde plus syringic acid.

a role in the early steps of the phenylpropanoid pathway that are common to flavonoid and sinapate ester biosynthesis.

Some *ref* mutations lead to changes in plant morphology: The *ref* and *brt* mutants were identified on the basis of their altered fluorescence under UV light. Although the *fah1-2* mutation completely eliminates sinapate ester production, it does not lead to any observable changes in plant growth, indicating that these compounds are dispensable for normal development (Chapple *et al.* 1992). In contrast, during the isolation of the *ref* and *brt* mutants, it became apparent that some of these mutations result in substantial changes in plant stature. The morphology of the *ref1*, *ref2*, and *brt1* rosettes is similar to the wild type, whereas *ref3-2* and *ref4-3* rosettes are reduced in size and display aberrant leaf shapes (Figure 1). After bolting, *ref1*, *ref2*, and *brt1* plants are morphologically similar to wild type while *ref3-2* and *ref4-1* plants are shorter (Figure 1) and are frequently more highly branched. Strong *ref3* alleles are also male sterile. Interestingly, the relative reduction in sinapoylmalate accumulation observed between the weak and strong *ref3* and *ref4* alleles (Figure 2) parallels the severity of the developmental phenotypes seen in these mutants.

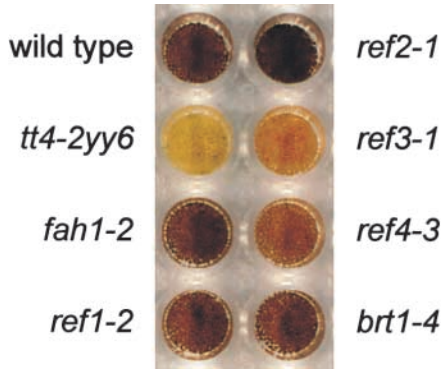

Figure 3.—Appearance of *brt1* and *ref* mutant seeds in comparison to wild type and the flavonoid-deficient *tt4* mutant.

DISCUSSION

The sinapate esters are a group of phenolic secondary metabolites whose distribution is generally restricted to the Brassicaceae. Although they are important UV protectants in Arabidopsis, they are dispensable under laboratory conditions. Since these compounds are fluorescent, they are readily visualized under UV light and thus make excellent reporters of phenylpropanoid pathway activity, both *in vitro* and *in vivo*. In addition, sinapoylmalate and sinapoylcholine are derived from a branch of the phenylpropanoid pathway that has not been the subject of many genetic studies. As a result, the isolation and analysis of mutants affected in sinapate ester biosynthesis promise to provide new insights into phenylpropanoid metabolism and its regulation.

The *BRT1* and *REF* genes are required for sinapate ester accumulation: The phenotypes of the mutants identified in this study (Table 5) indicate that the *BRT1* and *REF* genes are required for sinapoylmalate accumulation in leaves. Furthermore, the *REF1*, *REF3*, and to a lesser extent, *BRT1* genes are also required for sinapoylcholine biosynthesis in developing seeds. In contrast to strong *fah1* alleles, which eliminate sinapate ester biosynthesis in both leaves and seeds, the *brt1* and *ref* mutations cause only partial reductions in sinapate esters. This observation would suggest that other genes are partially functionally redundant with the *REF* and *BRT* genes. In this respect, it should be noted that the *ref1* and *brt1* mutants were recovered at relatively high

TABLE 5

Summary of *ref* and *brt* mutant phenotypes

	Sinmal[a]	Sincho[b]	Lignin	Flavonoids	Other
ref1	↓	↓			
ref2	↓		↓ in *S* units		
ref3	↓	↓	↓	↓	Dwarf, increased branching, male sterile
ref4	↓		↓	↓	Dwarf, increased branching
brt1	↓				Hyperfluorescent trichomes

[a] Sinapoylmalate.
[b] Sinapoylcholine.

frequencies, despite the subtle phenotypes that they display. Considering that these mutations do not appear to have deleterious effects on plant health, we suggest that *REF1* and *BRT1* are not essential genes and that some of the alleles identified are null. If so, other proteins must share functional roles with the *REF1* and *BRT1* gene products in the accumulation of wild-type levels of sinapate esters. An alternative explanation for the incomplete loss of sinapate esters in the *ref* and *brt1* mutants is that all the alleles recovered were leaky. This may be the case with the *ref3* and *ref4* mutants, since despite their relatively robust UV phenotypes, a total of only six of these mutants were identified in a screen of 100,000 seedlings. Given the dramatic effect of the stronger *ref3* and *ref4* mutations on plant development, and the male sterility of strong *ref3* mutants, it is tempting to speculate that the alleles we recovered were hypomorphic and that *REF3* and *REF4* are essential for embryo or seedling development, and that some *REF3* function is required for male fertility.

Considering that all of the mutants we identified carry recessive alleles and accumulate less sinapoylmalate than the wild type, it is likely that the *BRT1* and *REF* genes all have positive roles in phenylpropanoid metabolism, encoding enzymes or regulatory factors of the pathway. Alternatively, some of the *BRT1* or *REF* gene products may repress negative regulators of phenylpropanoid metabolism. The recent characterization of the Arabidopsis myb-like transcription factor AtMYB4 as a negative regulator of cinnamate 4-hydroxylase (C4H) expression indicates that regulatory mechanisms of this type are involved in the control of this pathway (Jin *et al.* 2000). Finally, we cannot exclude the possibility that the genes identified in this study encode negative regulators of sinapate ester degradation. Although sinapate ester levels decrease as Arabidopsis plants mature (Chapple *et al.* 1992), nothing is known about their metabolic fate.

The phenotypes of some of the mutants identified in this study suggest that the wild-type genes function in complex regulatory pathways controlling the developmental and tissue-specific accumulation of sinapate esters. For example, *ref2* mutants contain lower levels of sinapoylmalate in leaves, suggesting that *REF2* may be a positive regulator of phenylpropanoid metabolism in leaves. In contrast, the absence of sinapoylglucose and the presence of a compound that cochromatographs with sinapoylmalate in *ref2* seed extracts may indicate that *REF2* functions directly or indirectly as a negative regulator of the leaf-specific pathway of sinapate ester biosynthesis during embryonic development. Since sinapoylglucose is the precursor of sinapoylmalate in the reaction catalyzed by sinapoylglucose:malate sinapoyltransferase (SMT; Lorenzen *et al.* 1996; Lehfeldt *et al.* 2000), it is possible that this enzyme is aberrantly expressed in *ref2* embryos, leading to the conversion of sinapoylglucose to sinapoylmalate. Another such example is the combination of phenotypes observed in *brt1* plants. Although *brt1* mutants share the reduced epidermal fluorescence phenotype of the *ref* mutants, the most striking characteristic of *brt1* mutants is their hyperfluorescent trichomes. These two phenotypes are difficult to reconcile. The decreased levels of sinapoylmalate in *brt1* plants suggests that the *BRT1* gene activates phenylpropanoid biosynthesis, whereas the *bright trichomes* phenotype suggests that BRT1 normally represses the synthesis of sinapate esters in trichomes. Thus, BRT1 might be a protein with divergent regulatory functions in epidermal and trichome cells. An alternative, but more complex, explanation would be that the BRT1 protein might be required for the transport of sinapate esters into epidermal vacuoles. In the absence of BRT1 function, sinapate esters would be more available for diffusion or transport into trichomes, leading to the *bright trichomes* phenotype. At the same time, the failure of *brt1* plants to properly compartmentalize sinapate esters within the vacuole might lead to their enhanced degradation within the cytoplasm of epidermal cells and the *reduced epidermal fluorescence* phenotype also exhibited by *brt1* mutants.

A subset of *REF* genes is required for normal lignin biosynthesis: The *ref3* and *ref4* mutations decrease total lignin content, whereas *ref2* mutations decrease lignin syringyl monomer content while leaving total lignin deposition unchanged. These findings indicate that in addition to their role in sinapoylmalate and sinapoylcholine biosynthesis in leaves and seeds, the *REF2*, *REF3*, and *REF4* genes must be required for lignin biosynthesis

in stem tissue. In angiosperms, lignin is synthesized by the oxidative coupling of two types of monomers (Campbell and Sederoff 1996). Guaiacyl lignin, the major lignin component of Arabidopsis stems, is derived from polymerization of coniferyl alcohol, whereas syringyl lignin is synthesized from sinapyl alcohol. Until recently, it was thought that sinapyl alcohol, like sinapoylmalate, was synthesized from sinapic acid. It now seems more likely that coniferaldehyde and coniferyl alcohol are converted to sinapaldehyde and sinapyl alcohol by the concerted action of ferulate 5-hydroxylase (F5H) and caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (COMT; Humphreys *et al.* 1999; Osakabe *et al.* 1999). The *FAH1* locus encodes F5H, and *fah1* mutations decrease or eliminate syringyl lignin biosynthesis, but have little or no impact on total lignin content (Chapple *et al.* 1992; Meyer *et al.* 1998). Similarly, lignin syringyl monomer content is decreased in mutants defective in the COMT gene, such as the maize *bm3* mutant (Vignols *et al.* 1995), and in transgenic plants in which COMT has been downregulated (Atanassova *et al.* 1995; Van Doorsselaere *et al.* 1995; Lapierre *et al.* 1999). In contrast, transgenic plants in which upstream genes such as cinnamyl CoA reductase (CCR), cinnamyl alcohol dehydrogenase (CAD), or caffeoyl CoA *O*-methyltransferase (CCoAOMT) are downregulated deposit lower levels of lignin that often contain unusual monomers (Halpin *et al.* 1994; Baucher *et al.* 1996; Piquemal *et al.* 1998; Zhong *et al.* 1998). Plants carrying genetic lesions in some of these upstream genes have been identified and exhibit similar phenotypes (Ralph *et al.* 1997; Halpin *et al.* 1998).

The phenotypes of the mutant and transgenic plants described above suggest the *REF3* and *REF4* genes are likely to act upstream of *F5H* and thus have no impact on lignin monomer composition. These genes could encode phenylpropanoid pathway enzymes or regulators of their expression. The latter possibility seems more likely given the normal accumulation of sinapoylcholine in seeds of *ref4* mutants. In contrast, the phenotype of *ref2* plants suggests that REF2 may be required for normal expression or activity of F5H and/or COMT. Since F5H and COMT are thought to be required for the biosynthesis of all sinapate esters, this hypothesis appears to conflict with the observation that *ref2* mutants contain wild-type levels of sinapoylcholine. This phenotype could indicate that REF2 function is redundant in developing embryos. An alternative model for REF2 function is related to the observation that the F5H promoter requires the presence of a regulatory domain downstream of the F5H open reading frame for expression in leaves and stems (Ruegger *et al.* 1999). This 3′ domain is not required for expression in embryos. According to this model, *REF2* encodes a polypeptide, possibly a DNA-binding protein, required for the function of the *F5H* downstream regulatory domain. In the absence of the REF2 function, leaf sinapate ester and stem syringyl lignin biosynthesis would be compromised due to a decrease in *F5H* expression, whereas the accumulation of sinapoylcholine would be unaffected.

Two *REF* genes function in flavonoid biosynthesis: In Arabidopsis, *transparent testa* (*tt*) mutations lead to altered seed coat pigmentation. These mutants are defective in flavonoid biosynthesis, and the reduced content of flavonoid pathway-derived condensed tannins in their seed coats causes *tt* seeds to be less pigmented than wild type, varying from pale brown to bright yellow in color (Shirley *et al.* 1995). A common pathway provides precursors for sinapate ester, lignin, and flavonoid biosynthesis. Mutations in genes acting at or before the level of *p*-coumaric acid synthesis might lead to reductions in flavonoids as well as lignin and sinapate esters. In contrast, mutations acting after the synthesis of *p*-coumaric acid would be expected to affect both sinapate ester and lignin biosynthesis, but should not alter flavonoid metabolism. Both the *ref3* and *ref4* mutants exhibit sinapate ester-, flavonoid-, and lignin-deficient phenotypes, suggesting that REF3 and REF4 are likely to act early in the phenylpropanoid pathway. Alternatively, these phenotypes could be explained by metabolic "cross talk" between the flavonoid and sinapate ester pathways as has previously been documented in the *tt4* and *tt5* mutants (Li *et al.* 1993).

Mutations in the *REF3* and *REF4* genes alter plant growth and development: Most of the mutants in this study appear normal with respect to their growth and development. This observation suggests that the roles of the *REF1*, *REF2*, and *BRT1* genes may be limited to phenylpropanoid biosynthesis. In contrast, the altered development of the *ref3* and *ref4* mutants suggests that these genes may have multiple roles in directly regulating plant development and metabolism. Alternatively, phenylpropanoid metabolism downstream of REF3 and REF4 may lead not only to end products such as sinapate esters, flavonoids, and lignin, but also to metabolites required for normal plant growth. These compounds could include the dehydrodiconiferyl glycosides, compounds with cytokinin-like activity (Binns *et al.* 1987; Lynn *et al.* 1987; Teutonico *et al.* 1991; Orr and Lynn 1992) that recently were implicated in developmental aberrations seen in plants in which phenylpropanoid biosynthesis was downregulated (Tamagnone *et al.* 1998). Finally, the inability to metabolize phenylpropanoid precursors past the steps catalyzed or regulated by REF3 and REF4 may result in the accumulation of pathway intermediates that have deleterious effects on normal development.

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