The Plant Cell, Vol. 4, 1413–1424, November 1992

An Arabidopsis Mutant Defective in the General Phenylpropanoid Pathway

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Mutants of Arabidopsis deficient in a major leaf phenylpropanoid ester, 2-*O*-sinapoyl-L-malate, were identified by thin-layer chromatographic screening of methanolic leaf extracts from several thousand mutagenized plants. Mutations at a locus designated *SIN1* also eliminate accumulation of the sinapic acid esters characteristic of seed tissues. Because of increased transparency to UV light, the *sin1* mutants exhibit a characteristic red fluorescence under UV light, whereas wild-type plants have a blue-green appearance due to the fluorescence of sinapoyl malate in the upper epidermis. As determined by in vivo radiotracer feeding experiments, precursor supplementation studies, and enzymatic assays, the defect in the *sin1* mutants appears to block the conversion of ferulate to 5-hydroxyferulate in the general phenylpropanoid pathway. As a result, the lignin of the mutant lacks the sinapic acid-derived components typical of wild-type lignin.

INTRODUCTION

Phenylpropanoid metabolism in higher plants has been implicated in disease resistance (Hahlbrock and Scheel, 1989), UV light resistance (Caldwell et al., 1983), Agrobacterium *vir* gene activation (Stachel et al., 1985; Spencer and Towers, 1988), and plant growth regulation (Lynn et al., 1987) and is required for suberin and lignin biosynthesis. Mutants have been isolated in various plant species that affect the biosynthesis of flavonoids and anthocyanins; in Arabidopsis, mutants representing 11 *transparent testa* (*tt*) loci have been identified which are defective in various aspects of flavonoid biosynthesis (Koornneef, 1990). By contrast, there are no previous reports of mutations in Arabidopsis affecting another major class of soluble phenylpropanoid compounds, the hydroxycinnamic acid esters (Wintersohl et al., 1979).

Other crucifers, such as radish (Linscheid et al., 1980), accumulate the sinapic acid esters sinapoyl malate, which is found in leaf tissue, and sinapoyl choline (sinapine) and sinapoyl glucose, which are typically accumulated in seed tissue. Sinapoyl glucose serves as a common intermediate in the biosynthesis of both sinapoyl malate and sinapoyl choline, as shown in Figure 1 (Strack, 1977). The roles of these compounds are unclear. The hydrolysis of sinapoyl choline during germination of radish seeds has been shown to serve as a source of choline for the developing seedling (Strack, 1981), although it is not clear whether this reserve is essential. In addition, these esters and related phenylpropanoids have been suggested to play a role in UV light resistance in plants because they have high extinction coefficients in the biologically damaging UV-B range and they are primarily accumulated in the epidermis of plant organs. The relative contribution to UV light resistance made by flavonoids and hydroxycinnamic acid esters has not been investigated.

Lignin, one of the polymeric products of the general phenylpropanoid pathway, strengthens plant tissues and is also thought to be an important component of some disease resistance mechanisms (Moerschbacher et al., 1990). Several genes involved in the biosynthesis of hydroxycinnamic acids and lignin, phenylalanine ammonia-lyase, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (OMT), and peroxidase have been reported to be induced by inoculation of Arabidopsis leaves with a bacterial pathogen (Dong et al., 1991). At the agricultural and industrial level, lignin is also of substantial economic importance because it is an extremely complex polymer that is difficult to degrade chemically or biochemically (Crawford, 1981). Its presence interferes with the chemical release of cellulose from wood during pulp and paper production, and the presence of lignin and related phenolics in animal forage decreases the digestibility and hence the nutritional value of plant material (Cherney et al., 1990; Jung et al., 1991). The monomer composition of lignin has also been shown to have an impact upon its degradation (Chiang et al., 1988). Because of these considerations, it would be advantageous to be able to genetically manipulate lignin quality and quantity in both crop and forest species (Whetten and Sederoff, 1991).

Despite its importance, the general phenylpropanoid pathway has been examined by the isolation and characterization of mutants that are incapable of catalyzing specific steps of the pathway in only a few cases (Kuć and Nelson, 1964; Lapierre et al., 1988). This paper describes the identification of the major soluble phenolic esters of Arabidopsis and the

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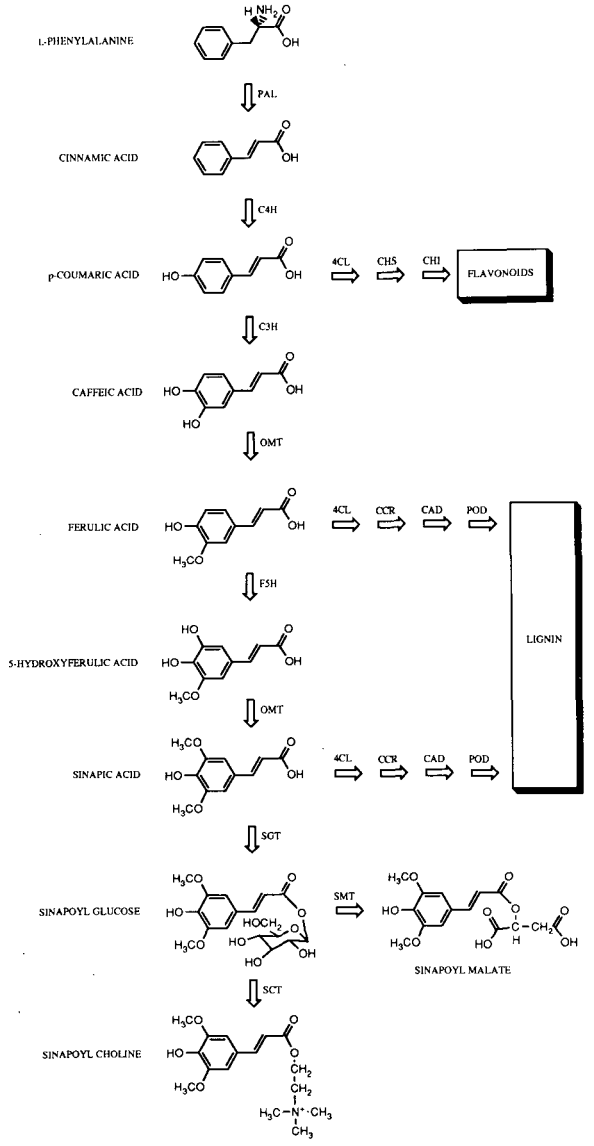

Figure 1. Scheme of Phenylpropanoid Metabolism in Arabidopsis.

The general phenylpropanoid pathway includes PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; C3H, *p*-coumarate-3-hydroxylase; OMT, caffeate/5-hydroxyferulate *O*-methyltransferase; and F5H, ferulate-5-hydroxylase. The enzymes responsible for the final synthesis of phenylpropanoid derivatives are SGT, sinapate: UDP-glucose sinapoyltransferase; SMT, sinapoyl glucose:malate sinapoyltransferase; SCT, sinapoyl glucose:choline sinapoyltransferase; 4CL, *p*-coumarate (hydroxycinnamate) coenzyme A ligase; CHS, chalcone synthase; CHI, chalcone-flavone isomerase; CCR, hydroxycinnamyl coenzyme A reductase; CAD, hydroxycinnamyl alcohol dehydrogenase; and POD, peroxidase.

isolation and characterization of mutants at a new locus designated *SIN1* (*SIN*apoyl malate biosynthesis). The *sin1* mutation causes a defect in the general phenylpropanoid pathway, consequently blocking the biosynthesis of sinapic acid esters, and altering the monomer composition of the plant's lignin. The availability of these mutants provides a novel opportunity to evaluate the physiological roles of these hydroxycinnamic acid esters and the impact of monomer composition upon the physicochemical characteristics of lignin.

RESULTS

Identification of Sinapic Acid Esters in Arabidopsis

Many members of the Brassicaceae are known to accumulate sinapic acid esters or related phenylpropanoid esters (Bouchereau et al., 1991). To identify the hydroxycinnamic acid esters found in Arabidopsis, the fluorescent compounds present in methanolic extracts of leaf and seed tissue were resolved by thin-layer chromatography (TLC) as shown in Figure 2, high-performance liquid chromatography (HPLC), and characterized by fast atom bombardment–mass spectrometry. As determined by TLC and HPLC, all of the fluorescent compounds cochromatographed with samples of authentic sinapoyl esters previously isolated and characterized from radish (Linscheid et al., 1980). Base hydrolysis of each ester (1 M NaOH for 1 hr at 37°C) yielded a single blue fluorescent band when analyzed by TLC; the band cochromatographed with authentic sinapic acid. Acid hydrolysis of the putative sinapoyl glucose followed by reduction, acetylation, and analysis by gas chromatography (GC) indicated the presence of only glucitol hexaacetate, which was obtained in 88% yield based upon the mass of sinapoyl glucose hydrolyzed. Fast atom bombardment–mass spectrometry was performed either directly on the isolated compounds (sinapoyl choline, positive ion; sinapoyl malate, negative ion) or on the acetylated derivative (sinapoyl tetracetyl glucose, positive ion). For all compounds tested, the expected major molecular ions were obtained and are given as follows: sinapoyl choline, 310; sinapoyl tetracetyl glucose, 554; and sinapoyl malate, 339. In addition, for sinapoyl malate, major ions were obtained for sinapic acid (223) and malic acid (133).

Taken together, the data indicate that the major blue fluorescent components of methanolic extracts of leaf and seed tissue of Arabidopsis are the sinapic acid esters 2-*O*-sinapoyl malate, 1-*O*-sinapoyl glucose, and sinapoyl choline.

Isolation and Characteristics of Mutants

Approximately 4200 randomly chosen ethylmethane sulfonate–mutagenized (M_2) plants were screened by TLC of methanolic leaf extracts for alterations in sinapoyl ester accumulation. The sinapoyl malate of wild-type leaves can easily be identified on thin-layer chromatograms of methanolic leaf extracts as a prominent blue fluorescent band under long wave UV light (Figure 2). Five independent mutant lines were identified that accumulated significantly lower levels of sinapoyl malate. Prior to further analysis, each mutant line was backcrossed

at least twice to the wild type and homozygous lines were established. Genetic complementation tests indicated that all of the mutations identified were allelic (data not shown). The mutations in the five lines were designated as alleles of a new locus, *SIN1*.

Leaf extracts of each mutant were analyzed by HPLC to quantitate the effect on sinapoyl malate accumulation (sinapoyl malate content [nmol g^{-1} fresh weight ± SE]: wild type, 2015 ± 295; *sin1-1*, 117 ± 10; *sin1-2*, 13 ± 3.0; *sin1-3*, 23 ± 2.3; *sin1-4*, 350 ± 30; *sin1-5*, 34 ± 7.0). The trace levels of sinapoyl malate measured by HPLC in the *sin1-2*, *sin1-3*, and *sin1-5* lines may represent other cochromatographing UV-absorbing extract components rather than residual sinapoyl malate because the latter is not detectable on thin-layer chromatograms of extracts from these mutants. Because the line carrying the *sin1-2* allele was almost completely deficient in sinapoyl malate and carried no visible deleterious background mutations, it was used for most subsequent studies.

Table 1. Sinapine and Choline Content of Defatted Seed Meal of Wild-Type and *sin1-2/sin1-2* Mutant Seed

Genotype	Sinapine (μmol g^{-1} defatted meal ± SE)	Choline (μmol g^{-1} defatted meal ± SE)	Total
+/+	24.8 ± 2.6	5.2 ± 0.29	30.0
sin1-2/sin1-2	1.4 ± 0.04	21.3 ± 0.58	22.7

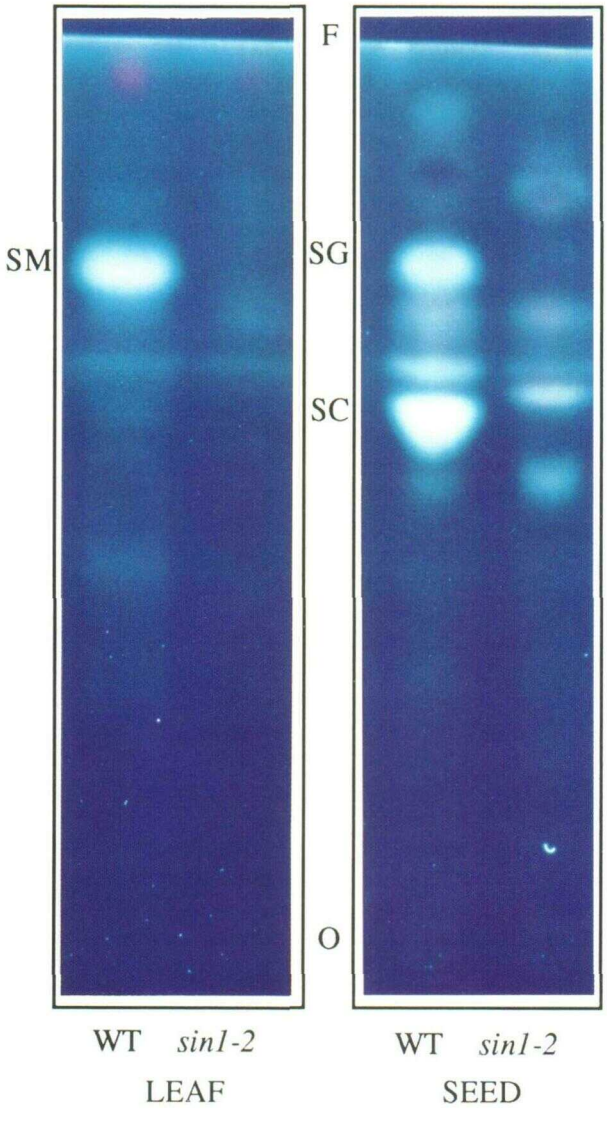

Figure 2. Thin-Layer Chromatograms of Leaf and Seed Extracts from the Wild Type and *sin1* Mutants.

Sinapic acid esters were extracted from wild-type (WT) and *sin1-2* leaves with 50% methanol, chromatographed, and visualized under UV light. The relative positions of sinapoyl malate (SM), sinapoyl glucose (SG), sinapoyl choline (SC), the sample origin (O), and solvent front (F) are indicated.

Measurement of the amount of sinapoyl malate in F_1 hybrids from crosses between the wild type and the *sin1* mutants indicated that all of the *sin1* mutations are recessive (the sinapoyl malate content of *sin1-2*/+ plants was 2090 ± 320 nmol g^{-1} fresh weight). Analysis of the segregation of the mutant phenotype in 144 F_2 progeny indicated that the *sin1-2* allele was inherited as a single Mendelian locus (105 *SIN1*/+, 39 *sin1/sin1*; $\chi^2 = 0.333$, $P > 0.05$). Analysis of the cosegregation of the *sin1* mutation with the phenotypic markers of the W100 mapping line (Koornneef et al., 1987) indicated linkage of the *sin1* mutation to the *CER2* locus on chromosome 4. Of 613 F_2 progeny from the cross, 148 were *sin1-2/sin1-2* homozygotes and 155 were *cer2/cer2* homozygotes (153 of each were expected given that each segregates as a single Mendelian locus). Only five *sin1/cer2* double mutants were recovered. These data place the *SIN1* locus ~18 centimorgans from the *CER2* locus on chromosome 4.

TLC analysis of seed extracts revealed that the *sin1* mutation affected the accumulation of sinapic acid esters throughout the plant because sinapoyl choline accumulation was also found to be reduced in the mutant (Figure 2). The sinapoyl choline levels in defatted seed meal of *sin1-2* was reduced to ~5% of wild-type levels as shown in Table 1. Increased levels of free choline in *sin1-2* seeds indicated that free choline accumulates in the mutant seed in place of the choline ester (Table 1).

Germination efficiency and early seedling growth was assessed for wild-type and mutant lines to determine whether the lack of sinapoyl choline in the seed influences early seedling development. The percent germination of mutant seed (99.3 ± 0.010) was indistinguishable from that of the wild type (98.9 ± 0.015). Similarly, there was no significant difference between the growth rates of the two lines, as can be seen in Figure 3, during the period of early seedling development when sinapoyl choline hydrolysis provides choline for phospholipid biosynthesis (Strack, 1981).

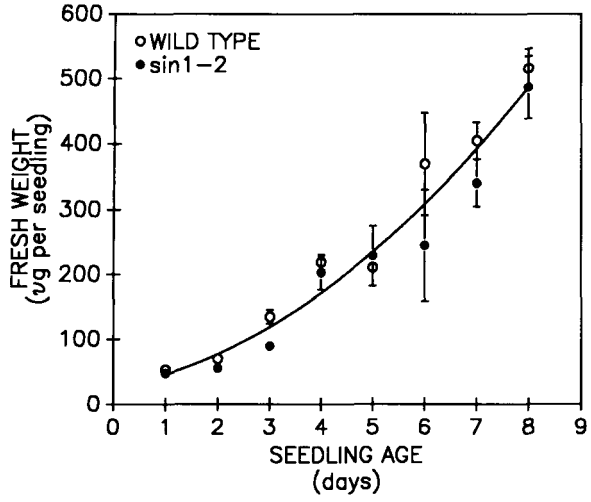

Figure 3. Growth Curve for Wild-Type and *sin1-2* Seedlings.

Fresh weight determinations were made on wild-type (○) and *sin1-2* (●) seedlings during the period of seedling growth when sinapoyl choline hydrolysis provides choline for phospholipid biosynthesis.

Sinapoyl Malate Fluctuations throughout Plant Development

To evaluate the relationship between plant development and sinapoyl ester metabolism, various plant tissues were sampled for sinapoyl malate content at various times throughout the growth cycle of wild-type and *sin1-2* plants. These results are shown in Figure 4. In wild-type leaf tissue, sinapoyl malate levels increased to a maximum of ~2 μmol g^{-1} fresh weight at week 3 of development, after which the levels gradually declined, except for a small increase (not statistically significant) just after the plants bolted. Very little sinapoyl malate was found in senescent leaf tissue (Figure 4), in rachis tissue, or in developing siliques (Figure 4 inset) and no sinapoyl malate was found in seeds (data not shown). Sinapoyl malate in *sin1-2* plants was reduced to levels only slightly above detectable limits at all stages of growth.

Characterization of the Biosynthetic Block in the *sin1* Mutants

To define the metabolic defect in the *sin1* mutants, the final enzymes of sinapate ester synthesis (Figure 1) were assayed in extracts of wild-type and mutant leaf tissue. No differences were found between mutant and wild-type extracts in the activity of sinapic acid:UDP-glucose glucosyl transferase (SGT), sinapoyl glucose:malate sinapoyl transferase (SMT), or sinapoyl glucose:choline sinapoyl transferase (SCT) (data not shown). These results suggested that the metabolic block existed in a step prior to the terminal enzymes of sinapoyl ester biosynthesis and was likely to be a block in the general phenylpropanoid pathway.

To examine the fate of phenylalanine in leaves of wild-type and *sin1* plants, individual leaves were excised and administered U-^{14}C-L-phenylalanine via the transpiration stream. After 2 hr, the leaves were harvested and extracted, and the extracts were saponified overnight in 0.1 M NaOH. This reaction frees all ester-bound phenolic acids, which together with previously unesterified (free) phenolic acids can be extracted into ether after acidification of the extract. These two pools (free and esterified) will henceforth be considered together; however, virtually identical results were obtained when the saponification step was omitted (data not shown), except that a significant pool of labeled sinapoyl malate was seen in wild-type chromatograms.

Two-dimensional TLC of the ether extracts from wild-type leaf incubations revealed significant incorporation of label into *p*-coumaric acid and sinapic acid, as shown in Figure 5A. Only low levels of radioactivity were seen in spots that cochromatographed with cinnamic acid and ferulic acid, and no label was seen associated with caffeic acid or 5-hydroxyferulic acid. Similar analysis of *sin1* samples showed significant differences in the fate of labeled phenylalanine (Figure 5B). No label cochromatographed with sinapic acid and there was a concomitant increase in label in ferulic acid. As in the wild type, no label was seen in the dihydroxyphenolic acids caffeic acid or 5-hydroxyferulic acid. Several other unidentified radiolabeled spots were found to increase significantly in labeling intensity in the extracts from the *sin1* leaf feedings.

To determine which of the two steps between ferulic acid and sinapic acid was blocked in the *sin1* mutants, surface-sterilized seedlings were grown on media containing 1-mM concentrations of various phenolic acids. Growth inhibition was apparent in seedlings grown on media containing ferulic acid.

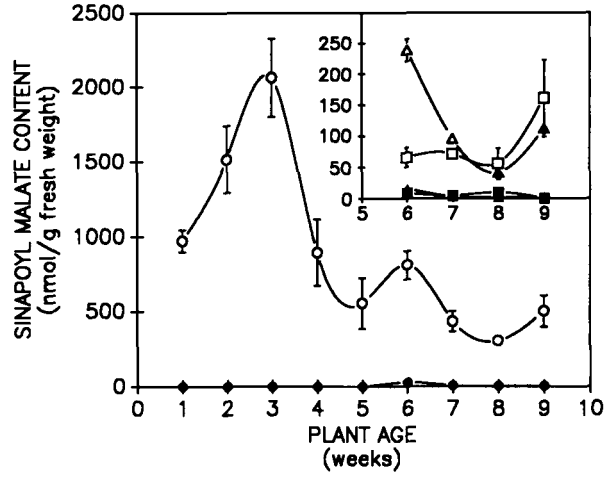

Figure 4. Accumulation of Sinapoyl Malate in Aerial Tissues of Wild-Type and *sin1-2* Arabidopsis.

The sinapoyl malate content of leaves (○, ●), rachis (△, ▲/inset), and siliques (□, ■/inset) of wild-type (open symbols) and *sin1-2* (filled symbols) plants were determined weekly, when possible.

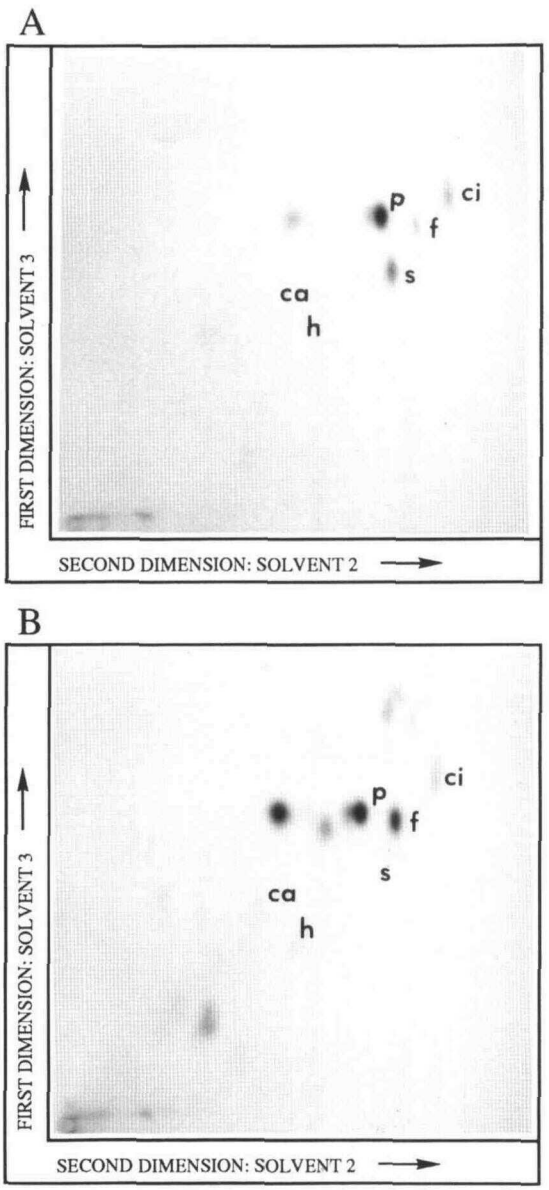

Figure 5. Hydroxycinnamic Acids Synthesized from Radiolabeled Phenylalanine in Arabidopsis Leaves.

U-^{14}C-L-phenylalanine was administered to leaves, and the radiolabeled products were extracted in methanol and partitioned into ether. Two-dimensional silica gel TLC, followed by autoradiography, was used to identify the radiolabeled metabolites synthesized. The positions of phenolic acids are labeled as follows: ci, cinnamic acid; p, *p*-coumaric acid; ca, caffeic acid; f, ferulic acid; h, 5-hydroxyferulic acid; s, sinapic acid.

(A) Phenolic acids labeled in wild-type leaves.

(B) Phenolic acids labeled in *sin1-2* leaves.

Seedlings grown on media containing 5-hydroxyferulic acid grew more vigorously than those on ferulic acid media, although their roots blackened, presumably due to the oxidation of this dihydroxyphenol catalyzed by extracellular peroxidases. The inclusion of phenolic acids in the media suppressed the accumulation of sinapoyl malate in wild-type seedlings, perhaps due to inhibition of phenylpropanoid pathway enzymes, or other nonspecific inhibitory effects. Sinapoyl malate did not accumulate in *sin1-2* seedlings grown on media containing ferulic acid. On the other hand, in the seedlings fed 5-hydroxyferulic acid or sinapic acid, there was a significant chemical complementation of the *sin1* phenotype as can be seen in Figure 6. TLC of base hydrolysates of extracts from *sin1-2* plants grown on 5-hydroxyferulate-containing media revealed the presence of sinapic acid in the reaction products, indicating that the exogenous 5-hydroxyferulate had been O-methylated to form sinapic acid.

Consistent with these observations, assays of caffeic acid/5-hydroxyferulic acid:*S*-adenosylmethionine OMT activity in wild-type and *sin1-5* extracts showed no significant difference between wild-type (activity in pkat mg^{-1} protein toward caffeic acid, 8.5; 5-hydroxyferulate, 12.4) and mutant tissue (activity in pkat mg^{-1} protein toward caffeic acid, 10.5; 5-hydroxyferulate, 15.8). This suggests that the activity of ferulate-5-hydroxylase is defective in the *sin1* mutant. Despite numerous attempts to assay ferulate-5-hydroxylase directly using both ^{14}C-ferulate and ^{14}C-feruloyl coenzyme A as substrates, and assaying the enzyme as a monooxygenase or as an ascorbate/α-ketoglutarate–dependent dioxygenase, the activity of the enzyme could not be demonstrated in either mutant or wild-type extracts.

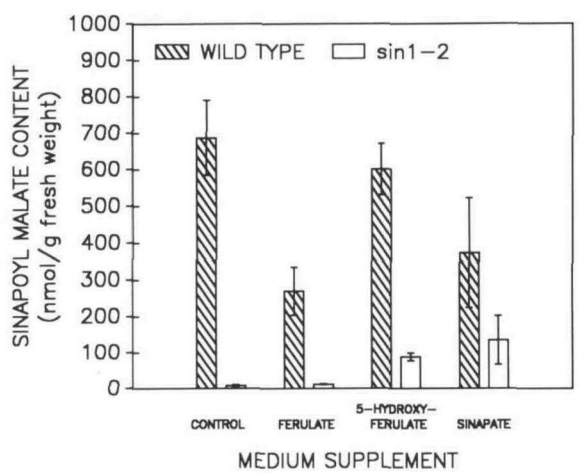

Figure 6. Chemical Complementation of the *sin1* Phenotype by Metabolite Supplementation.

Wild-type (hatched bars) or *sin1* mutant (open bars) seedlings were grown for 10 days on unsupplemented media or media containing 1 mM ferulic acid, 5-hydroxyferulic acid, or sinapic acid, and were harvested for HPLC analysis of sinapoyl malate content.

Lignin Analysis in Wild Type and *sin1* Mutants

To ascertain the effect of the *sin1* mutation on lignin monomer composition, wild-type and *sin1* mutant rachis tissue samples were subjected to nitrobenzene oxidation and GC analysis to determine their relative content of guaiacyl (derived from ferulic acid) and syringyl (derived from sinapic acid) units as vanillin and syringaldehyde, respectively. The ratio of vanillin to syringaldehyde released from wild-type lignin was found to be 2:1, whereas the amount of syringyl residues liberated from *sin1* lignin was below detection limits, as shown in Figure 7. The content of *p*-hydroxybenzaldehyde recovered from both wild-type or mutant rachis tissue oxidations was ~1% of the total phenolic aldehydes quantified. No 5-hydroxyvanillin, a potential degradation product from lignin containing 5-hydroxyferulic acid–derived units, was detected.

The lignin composition and distribution were determined histochemically in hand sections of fresh basal rachis segments using the Mäule reagent. Figure 8 shows that the sclerified parenchyma between the vascular bundles of wild-type tissue stained red with the reagent, indicating a positive reaction for the presence of syringyl units. By contrast, the mutant rachis segments gave a negative reaction. Interestingly, the vascular bundles of both wild-type and mutant tissue give a negative reaction with the Mäule reagent, indicating that the lignin in these tissues is primarily guaiacyl lignin.

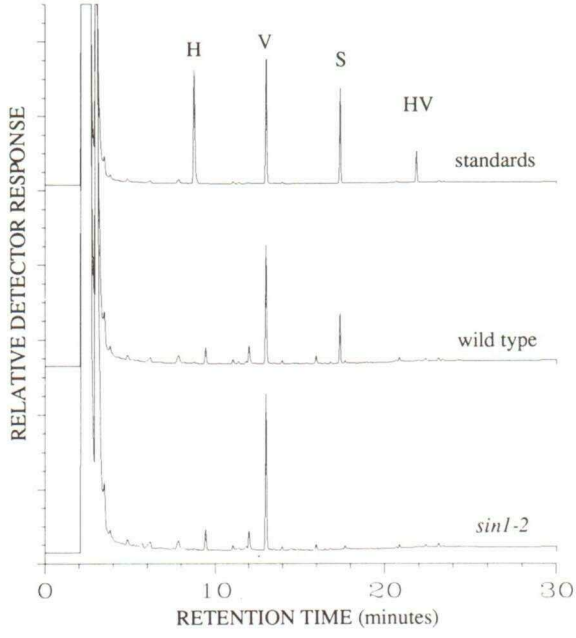

Figure 7. Gas Chromatograms of Nitrobenzene Oxidation Products of Wild-Type and *sin1-2* Rachis Lignin.

The relative positions of the derivatives of the expected lignin degradation products *p*-hydroxybenzaldehyde (H), vanillin (V), and syringaldehyde (S), as well as the derivative of the potential degradation product, 5-hydroxyvanillin (HV), are indicated on the upper chromatogram.

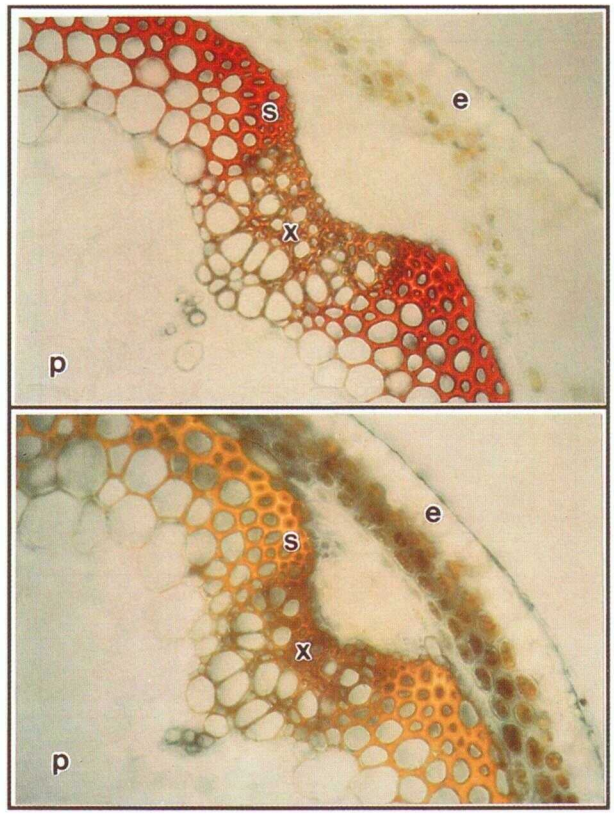

Figure 8. Histochemical Staining of Lignin in the Wild Type and *sin1-2* Mutants of Arabidopsis.

Hand sections of rachis tissue were fixed in glutaraldehyde and stained using the Mäule reagent. The positions of the pith (p), sclerified parenchyma (s), xylem (x), and epidermis (e) are indicated. Magnification × 50.

Appearance of Wild-Type and Mutant Plants under UV Light

It has been reported previously that sinapic acid esters are accumulated primarily in the epidermis of radish cotyledons (Strack et al., 1985). To determine whether the lack of an epidermal UV light–absorbing metabolite can be visualized in vivo, wild-type and *sin1* mutant plants were examined and photographed using a 365-nm transilluminator as a light source. As shown in Figure 9, wild-type plants exhibit a blue–green fluorescence under UV light (the color of the fluorescence is skewed toward the green due to the use of a pale yellow barrier filter to screen out reflected UV light during photography), whereas the *sin1* mutants exhibit red chlorophyll fluorescence.

This phenotype was observed in all of the independently derived *sin1* mutants and was also observed to cosegregate with the *sin1* mutation in F_2 populations scored for sinapoyl malate content by TLC of leaf extracts.

DISCUSSION

The genetic lesion in the *sin1* mutant leads to an array of biochemical phenotypes in a plant that is morphologically wild type. Although the *sin1* mutants were identified by screening for mutants unable to accumulate sinapoyl malate, the biochemical defect was not specific to sinapoyl malate biosynthesis but instead blocked sinapic acid synthesis generally, resulting in the absence of the main seed sinapic acid ester, sinapoyl choline (Figure 2). In germinating radish seedlings, this compound is hydrolyzed and the choline released is used for phospholipid biosynthesis (Strack, 1981). Because the mutant accumulated relatively high levels of free choline in seeds, it is clear that the synthesis of choline continues relatively unabated in the mutant, despite the fact that it is no longer utilized by sinapoyl glucose:choline sinapoyl transferase to form the sinapic acid ester (Table 1). If a seed choline reserve is, in fact, necessary for efficient germination and seedling growth, the pool of unesterified choline in the mutant seed may well satisfy this requirement. Interestingly, the presence of relatively high levels of sinapoyl choline in canola seed has a negative impact on the value of canola meal (Hobson-Frohock et al., 1977). Considering that the *sin1* mutants of Arabidopsis germinate and develop as rapidly as wild-type seedlings (Figure 3), it should also be possible to genetically eliminate sinapoyl choline from Brassica seeds without a deleterious effect on seedling growth.

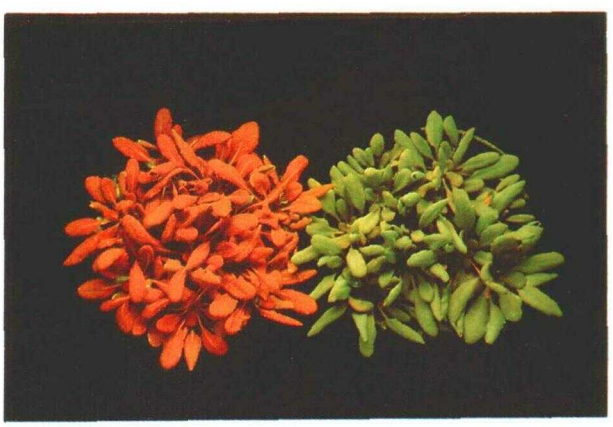

Figure 9. Wild-Type and *sin1* Mutant Plants Photographed under UV Light.

Wild-type (right) and *sin1* mutant (left) plants were photographed using a 365-nm transilluminator as a light source and a pale yellow barrier filter to remove reflected UV light. The green color of the wild-type plants is due to the fluorescence of sinapoyl malate in the leaves' upper epidermis and appears green rather than blue due to the barrier filter employed during photography. The red color of the mutant is due to UV-induced chlorophyll fluorescence that is revealed in the absence of the sinapoyl malate fluorescence.

The red appearance of the mutants under long wave UV illumination (Figure 9) is presumably due to chlorophyll fluorescence in the mesophyll that results from the lack of UV attenuation in the epidermis. By contrast, the fluorescence of sinapoyl malate gives wild-type plants a blue–green appearance (Figure 9). Experiments are currently underway to evaluate the relative contributions of sinapoyl malate and flavonoids to UV-B resistance in Arabidopsis utilizing the *sin1* and *tt4* mutants as well as *sin1/tt4* double mutants. Preliminary results indicate that the *sin1* mutants are significantly more sensitive to light in the UV-B range than the wild type (C.C.S. Chapple and R. Last, unpublished results). In addition, because the *sin1* mutation does not have an impact upon plant growth, the red fluorescent phenotype of the *sin1* mutants may make the mutant useful as a genetic marker in Arabidopsis research. For example, cloning of the *SIN1* gene may allow the construction of chimeric genes employing the *SIN1* coding sequence as a readily scored visible marker when transformed into a *sin1* mutant.

Initially, it seemed possible that the *sin1* mutation might affect the activity of sinapic acid:UDP-glucose sinapoyl transferase because the inability to synthesize sinapoyl glucose would prevent the synthesis of both sinapoyl malate in leaf tissue and sinapoyl choline in seed tissue. However, the activity of this transferase, as well as those of the other two enzymes devoted to sinapoyl malate and sinapoyl choline biosynthesis (SMT and SCT, respectively) were found to be identical in extracts of mutant and wild-type tissues. The feeding studies indicated that the defective enzymatic step in the mutant lies in one of the two enzymes responsible for the conversion of ferulic acid to sinapic acid in the general phenylpropanoid pathway. Although seedling growth on media containing phenolic acids was inhibited, and sinapoyl malate accumulation was generally depressed, growth of *sin1* mutant seedlings on media containing 1 mM 5-hydroxyferulic or sinapic acid partially restored sinapoyl malate accumulation. Exogenous ferulic acid did not suppress the mutant phenotype (Figure 6). These data are consistent with the hypothesis that the *sin1* mutation affects the activity of ferulic acid 5-hydroxylase. The lack of full complementation by exogenous phenolic acids may be due to poor uptake of these precursors, and/or to oxidation of 5-hydroxyferulate by cell wall–bound peroxidases. This oxidation may impede uptake directly or may reduce the effective concentration of the administered compound below the levels required to effect full chemical complementation.

The radiotracer feeding experiments clearly show the conversion of ^{14}C-phenylalanine to *p*-coumaric acid and sinapic acid, as well as a number of unidentified components in wild-type leaves. By contrast, in leaves of the *sin1-2* mutant, sinapic acid is not labeled and there is a concomitant accumulation of label in ferulic acid, clearly establishing that the *sin1* mutant is blocked in the general phenylpropanoid pathway. Although several of the intermediates in the pathway become

strongly labeled, in mutant and wild type no significant label is found to accumulate in caffeic acid or 5-hydroxyferulic acid. It may be that these two intermediates are rapidly channeled through the next biosynthetic step by caffeic acid/5-hydroxyferulic acid OMT, preventing the build-up of a significant radiolabeled pool. Nevertheless, in the mutant, the build-up of ferulic acid rather than 5-hydroxyferulic acid is consistent with a metabolic block in ferulate-5-hydroxylase, the subsequent biosynthetic step.

Although the radiotracer data do not rule out a defect in the O-methylation of 5-hydroxyferulate, the activity of OMT thought to be responsible for the conversion of 5-hydroxyferulic acid to sinapic acid is unaffected in extracts of mutant leaf tissue. In addition, the O-methylation of both caffeic acid to ferulic acid and 5-hydroxyferulic acid to sinapic acid has been previously reported to be catalyzed by a single bispecific enzyme (De Carolis and Ibrahim, 1989; Gowri et al., 1991; Bugos et al., 1992). If the gene encoding this bispecific OMT were the site of the genetic lesion in the *sin1* mutants, it would be expected that the biosynthesis of hydroxycinnamic acids in the mutants could not proceed past caffeic acid. The radiotracer feeding experiment data clearly show the conversion of ^{14}C-phenylalanine into ferulic acid in the mutant leaf tissue, suggesting that the bispecific OMT is an unlikely candidate for the biochemical block in the *sin1* mutants.

Although evidence indicates that the hydroxylation of ferulic acid is the blocked step in the *sin1* mutant, it has thus far proven impossible to assay in vitro this enzymatic activity in leaf extracts of wild type and *sin1* mutants. It is currently not clear why this enzyme should be intractable, because it has been assayed in extracts of other species (Grand, 1984); however, it may be present in Arabidopsis leaves at levels too low to assay in vitro. In contrast, assays of the bispecific OMT indicate that it is present in substantial amounts in leaf extracts. If these two observations accurately represent the situation in vivo: rate limiting synthesis of 5-hydroxyferulate and rapid O-methylation of 5-hydroxyferulate, they may explain the failure to recover radiolabeled 5-hydroxyferulic acid after ^{14}C-phenylalanine feeding experiments, even under conditions where sinapic acid (Figure 5A) becomes heavily labeled.

The build-up of ferulic acid in the mutant raises the question of why the mutant does not synthesize feruloyl glucose and subsequently, feruloyl malate. When assayed in vitro, Arabidopsis SGT, SCT, and SMT are all capable of catalyzing the synthesis of the respective feruloyl esters. The fact that the mutant does not accumulate any of these compounds may indicate that some form of compartmentalization or channeling prevents the diversion of the accumulated ferulic acid into the soluble ester pool.

A particularly interesting aspect of the *sin1* mutation is its impact on lignin biochemistry. As is commonly seen in dicotyledonous angiosperms (Lewis and Yamamoto, 1990), wild-type Arabidopsis synthesizes guaiacyl-syringyl lignin, containing both ferulic acid–derived guaiacyl units and sinapic acid–derived syringyl residues. Noncross-linked moieties of these types are liberated from lignin by nitrobenzene oxidation as vanillin and syringaldehyde, respectively (Lewis and Yamamoto, 1990) (Figure 7). Consistent with the impact of the *sin1* lesion on the soluble ester pool, the mutants are incapable of synthesizing the syringyl units present in wild-type lignin. This result was confirmed histochemically by use of the Mäule reagent, which yields a red (positive) reaction with syringyl lignin. In hand sections of wild-type tissue, but not in *sin1* mutant tissue, this results in staining of the sclerified parenchyma that develops between the vascular bundles (Figure 8), thus confirming the results obtained by GC analysis of the products of nitrobenzene oxidation. Interestingly, the vascular bundles do not show a positive reaction for the presence of syringyl lignin, indicating a developmental relationship between lignification and lignin monomer composition. It has been observed previously that lignin from juvenile tissue, such as primary xylem, is lower in syringyl moieties than lignin from sclerenchyma and secondary xylem (Venverloo, 1971). It is interesting that in Arabidopsis and other crucifers, the genes required for sinapic acid biosynthesis are expressed in developing seed tissue, leaf epidermis, and rachis sclerenchyma, three tissues that are developmentally unrelated.

The chemical degradation of lignin is an industrial process of significant importance that is expensive and potentially hazardous to the environment, and the degradability of lignin is thought to be directly related to its monomer composition. The presence of 5-*O*-methyl groups in syringyl lignin is thought to decrease cross-linking opportunities in the polymer, resulting in syringyl lignin being easier to pulp for paper production than guaiacyl lignin (Chiang et al., 1988). Similarly, the presence of lignin in animal fodder significantly reduces its digestibility by reducing access of hydrolytic enzymes to cell wall polysaccharides.

The *sin1* mutant of Arabidopsis offers the opportunity to examine the impact of lignin monomer composition on the physicochemical properties of lignin in an otherwise isogenic background. It may also facilitate the cloning of the *SIN1* gene by map-based cloning methods. The *SIN1* gene may encode ferulate 5-hydroxylase, one of the genes required for the synthesis of syringyl lignin that has yet to be cloned. This is presumably due to the difficulty in purifying what is thought to be a membrane-bound protein. The cloning and manipulation of this gene may also allow the modification of lignin synthesis in existing species or the introduction of syringyl lignin into species that do not currently synthesize it. Although other steps in the lignin biosynthetic pathway may influence lignin monomer composition (Nakamura et al., 1974; Grand et al., 1983), the introduction or alteration of expression of ferulate-5-hydroxylase may be an important factor in modifying lignification in economically important plants.

METHODS

Plant Material

All experiments were performed using *Arabidopsis thaliana* ecotype Columbia cultivated under continuous light at a light intensity of 100

μmol m^{-2} sec^{-1} at 23°C, except for the radiotracer feeding experiments where plants were grown under a photoperiod of 8-hr light/16-hr dark under otherwise similar conditions. All physiological experiments were performed on plants that had been backcrossed to the wild type and reselected from segregating F_2 populations at least twice, and in most cases four times, to remove nonlinked background mutations.

Chromatography

For analysis of intact sinapic acid esters by silica gel thin-layer chromatography (TLC), a mobile phase of *n*-butanol/glacial acetic acid/water (5:2:3; solvent 1) was employed. For analysis of free phenolic acids, the mobile phase used was toluene/glacial acetic acid 2:1 saturated with water (solvent 2). For two-dimensional TLC, plates were run first in petroleum ether/ethyl acetate/methanol/acetic acid (10:10:1:0.2; solvent 3) and then in solvent 2. In all cases, compounds were visualized under long wave UV light (365 nm) by their characteristic fluorescence (caffeic acid, ferulic acid, 5-hydroxyferulic acid, sinapic acid, and their esters) or under shortwave UV light (254 nm) by their characteristic absorbance (cinnamic acid and *p*-coumaric acid).

For high-performance liquid chromatography (HPLC) analysis, sinapic acid esters and free phenolic acids were injected on a Nucleosil C-18 column, and eluted using a gradient from 2% aqueous acetic acid to 2% acetic acid in tetrahydrofuran/acetonitrile/water 4:4:2. Quantitation of compounds was performed based on UV light absorbance at 330 nm using authentic compounds isolated from *Raphanus sativus* (kindly provided by Dr. Dieter Strack) or phenolic acids purchased from Sigma.

Isolation of Compounds

The isolation of the putative sinapoyl esters was accomplished by a combination of ion exchange and reverse phase column chromatography, and was monitored by spotting samples of column fractions on paper to identify those that contained compounds which exhibited blue fluorescence under long wave (365 nm) UV light.

For the isolation of sinapoyl glucose and sinapoyl choline, a 10-g sample of seeds of wild-type Arabidopsis ecotype Columbia was thoroughly ground in a mortar with 10 mL of hexane. The hexane phase was decanted and the tissue was reground with an additional 10 mL of hexane. The defatted seed meal was filtered off using Whatman No. 1 filter paper and was allowed to air dry. The meal was then extracted twice for 30 min with 200 mL of cold 50% methanol. The residual meal was filtered off and the methanolic extracts were pooled, dried in vacuo, redissolved in 10 mL of water, and passed through a 10 × 5 cm inner diameter (i.d.) column of Sephadex QAE-A-50. Fractions of interest were pooled, dried in vacuo, redissolved in 10 mL of water, and applied to a 15 × 5 cm i.d. column of Sephadex SP-C-50. Neutral compounds were first eluted from the column with water and fractions of interest were again pooled, dried in vacuo, and redissolved in 5 mL of water. Cationic compounds were eluted from the column with 0.2 M NH_4OH and fractions of interest were similarly identified, pooled, dried, and redissolved. For both classes of compounds, final purification was achieved by application of the redissolved material onto Waters C-18 SepPak cartridges, previously washed with methanol and re-equilibrated with water. In each case, the mixture was applied and unbound material was washed through with water; bound compounds were eluted with a step gradient of methanol/water.

In the fractionation of the neutral compounds, the sinapoyl glucose was eluted in the 5, 10, and 20% methanol fractions. These three fractions were concentrated to dryness in vacuo and the sinapoyl glucose was recrystallized from water to yield colorless needles. To identify the sugar moiety of the sinapoyl ester, a sample was hydrolyzed at 80°C for 3 hr in 1 M H_2SO_4. The samples were then neutralized with 9 M NH_4OH and reduced (1 mL of 2% sodium borohydride in DMSO, 90 min at 40°C), acetylated (4 mL of acetic anhydride plus 250 μL of 1-methylimidazole as catalyst, 10 min at room temperature), extracted into methylene chloride, and analyzed by gas chromatography (GC). All samples were analyzed using an HP 5890 gas chromatograph equipped with a Supelco 30 m × 0.75 mm i.d. SP-2330 column and flame ionization detection.

From the cationic fraction, sinapoyl choline was retained only slightly on the reverse phase resin, and eluted in the later aqueous washes and 5% methanol fractions. This material was pooled, concentrated, and was subsequently judged to be >95% pure by TLC, using nonspecific staining methods (iodine vapor, sulfuric acid charring).

For the isolation of sinapoyl malate, leaves of wild-type Arabidopsis ecotype Columbia (85 g) were extracted in 1 L of 50% methanol overnight at 4°C. The extract was filtered through cheesecloth, concentrated to dryness in vacuo, and redissolved in 10 mL of water. The solution was clarified by centrifugation and applied to a 24 × 5 cm i.d. column of Sephadex QAE-A-50. The column was washed with water and bound material was eluted with 4% formic acid. Fractions of interest were pooled, dried in vacuo, redissolved in 4% formic acid, and applied to a Waters C-18 SepPak. Unbound material was eluted with water and bound materials were eluted with a step gradient of methanol. Sinapoyl malate eluted in the fractions containing 10, 20, and 30% methanol. These fractions were pooled, dried in vacuo, redissolved in water, and lyophilized.

Genetic Methods

For mutant screening, leaves of individual M_2 plants from an ethylmethane sulfonate–mutagenized population were extracted for 30 min in 50 μL of 50% methanol. Five microliters of the extract was analyzed by TLC for the presence of sinapoyl malate. Putative mutants were retested in the next generation and plants showing heritable changes in their sinapoyl malate levels were backcrossed to the wild type.

For complementation analyses, reciprocal crosses of each mutant line were made to *sin1-2* plants and to the wild type. Leaf material was harvested when plants were 3 weeks of age and was extracted in 100% methanol. The methanolic extracts were dried in vacuo, redissolved in 2% acetic acid, and analyzed by HPLC.

To map the *sin1* locus, a cross was performed using a *sin1-2* homozygote as a female parent and the Wageningen marker line W100 (Koornneef et al., 1987) as the pollen donor. The segregation of the *sin1* phenotype and the morphological markers of the W100 line, with the exception of *ms-1*, was scored in the F_2 progeny.

Assay of Free Choline Content of Seeds

Choline was assayed according to the protocol described in Takayama et al. (1977). Briefly, defatted seed meal preparations of wild-type or *sin1-2* seed were prepared as described above. Aqueous extracts of the defatted seed meal were prepared by heating meal samples of 100 mg in 4 mL of water at 100°C for 30 min in a sealed tube. Choline assay reagent was freshly prepared as follows: 10 mL 50 mM Tris-HCl, pH 7.8, containing 1.2 mg 4-aminoantipyrine, 2 mg phenol, 0.8 mg $CaCl_2 \cdot 2H_20$, 25 units horseradish peroxidase, and 10 units choline

oxidase. Reactions were conducted for 30 min at 30°C by combining up to 10 μL of sample with 90 μL of color reagent. After 30 min, the absorbance of the samples was measured at 500 nm.

Measurement of Seedling Growth Rate

Seedling growth rate was determined by growing seedlings on nutrient media covered with nitrocellulose membranes so that seedlings could easily be removed from the membranes without adherent agar. Sterile nitrocellulose membranes (BA85; Schleicher & Schuell) were placed on Murashige-Skoog medium (Murashige and Skoog, 1962) (Gibco) containing 1% sucrose and 0.8% agar. Surface-sterilized seeds of the wild type and *sin1-2* were sown on the nitrocellulose and the closed plates were incubated at 22°C with continuous fluorescent illumination (25 μmol m^{-2} sec^{-1}). The seeds on each filter were counted, and every 24 hr, replicate samples of seedlings were removed from the nitrocellulose and weighed.

Radiotracer Feeding Experiments

Individual leaves of wild-type and mutant plants were excised under water and placed with their petioles immersed in a solution containing 7.4 × 10^4 Bq U-^{14}C-L-phenylalanine (specific activity: 14.2 GBq $mmol^{-1}$) in 40 μL of water. After a 2.5-hr incubation period at a light intensity of 100 μmol m^{-2} sec^{-1}, the leaves were removed, transferred to a microcentrifuge tube containing 1 mL of methanol and a mixture of phenolic acids (40 μg each of cinnamate, *p*-coumarate, caffeate, ferulate, 5-hydroxyferulate, and sinapate), and heated for 20 min at 60°C. The crude extracts were concentrated to dryness in a stream of N_2 at 30°C and redissolved in 40 μL of methanol to which was then added 100 μL of argon-sparged 0.1 M KOH. After hydrolysis overnight at 37°C, 100 μL of 1 M HCl was added to each sample, and the samples were extracted twice with 500 μL of ether. The ether phases were pooled, dried in a N_2 stream, and redissolved in 50 μL of methanol. Samples of all extracts representing ~20,000 dpm were analyzed by two-dimensional TLC and the distribution of radioactivity was assessed by autoradiography.

Metabolite Supplementation

Murashige-Skoog plates (1% sucrose, 0.8% agar, pH 5.6) were prepared containing 1 mM cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid, 5-hydroxyferulic acid, or sinapic acid (all previously dissolved in DMSO; final DMSO concentration of 0.4%). Control plates contained only 0.4% DMSO, which was found to have no visible impact on either seedling growth or sinapoyl ester accumulation, as determined by HPLC (data not shown). Seeds of the wild type and *sin1-2* were surface sterilized, sown directly on the agar surface, and cultivated as described above. After 10 days of growth, methanolic seedling extracts were analyzed by HPLC.

Enzyme Assays

Aseptically grown 5-day-old seedlings were harvested for the assay of sinapic acid:UDP-glucose sinapoyl transferase (SGT), sinapoyl glucose:choline sinapoyltransferase (SCT), and sinapoyl glucose:malate sinapoyltransferase (SMT). Seedlings (350 mg) were ground in liquid nitrogen and stirred for 30 min in 3 mL of 100 mM potassium phosphate buffer (SMT: pH 6.0, SCT: pH 7.0, SGT: pH 7.0 plus 20 mM β-mercaptoethanol) in the presence of 4% w/v polyvinylpolypyrrolidone. The extracts were centrifuged for 5 min at 10,000*g*, and desalted into extraction buffer on Pharmacia PD-10 columns. Assays were conducted in a final volume of 160 μL for 30 to 60 min at 30°C in the presence of 1.25 mM ferulic acid, 5-hydroxyferulic acid, or sinapic acid and 1.25 mM UDP-glucose (SGT), or 1.25 mM feruloyl glucose, or sinapoyl glucose plus 60 mM L-malate (SMT), or 2.5 mM choline chloride (SCT). Incubations were terminated by the addition of 160 μL of acetonitrile, and reaction products were quantified by HPLC as described above.

Extracts for assay of caffeate/5-hydroxyferulate *O*-methyltransferase (OMT) were prepared essentially as described previously (Edwards and Dixon, 1991). One-gram samples of wild-type or *sin1-5* tissue were ground with a glass homogenizer in 4 mL of 200 mM Tris-HCl, pH 7, containing 14 mM 2-mercaptoethanol. Samples were centrifuged for 5 min at 10,000*g*, and the supernatants were then brought to 80% saturation of ammonium sulfate, incubated on ice for 20 min, and centrifuged for 20 min at 10,000*g*. The pellets were redissolved in a minimal volume of extraction buffer and desalted into the same buffer. Assays were conducted in a final volume of 100 μL, containing 30 μM methyl-^{14}C-*S*-adenosyl methionine (700 Bq per assay) and 500 μM caffeic acid and/or 5-hydroxyferulic acid (each added in 5 μL of DMSO; all assays were brought to a final concentration of 10% DMSO). Assays were incubated for 30 min at 37°C, then stopped by the addition of 50 μL of a mixture of 5 mM ferulic acid and 5 mM sinapic acid in absolute ethanol, and 100 μL of 1 M HCl. Assays were extracted with 1 mL of ether and centrifuged; 100 μL was assayed by liquid scintillation counting and the remainder was dried, redissolved in 15 μL of methanol, and fractionated by silica gel TLC (solvent 2). The position of the radioactive products was determined using a Bioscan TLC plate scanner.

Protein content was determined according to Bradford (1976) using bovine γ-globulin as a standard.

Lignin Analysis

Samples of dried rachis tissue were ground in a Wiley mill to pass a 1-mm mesh and were processed for nitrobenzene oxidation as described previously (Galletti et al., 1989). Ground material (50 mg) was extracted three times with 1 mL of methanol and twice with water (both at 60°C). Esterified phenolics were extracted by saponification for 60 min at 37°C with 1 M NaOH and the material was rinsed twice and resuspended in water. An aliquot equivalent to 20 mg dry weight of ground material was brought to 2 M NaOH in a screw cap tube to which was added 20 μL of nitrobenzene (final volume 1 mL) and the tubes were sealed and incubated at 160°C for 2 hr. Samples were allowed to cool and were neutralized by diluting 250 μL into 750 μL of 1 M acetic acid; 100 μL of the neutralized sample was reduced, acetylated, and extracted into methylene chloride as described above for the isolation of sinapoyl glucose. As standards, samples containing 20 μg of *p*-hydroxybenzaldehyde, vanillin, 5-hydroxyvanillin, and syringaldehyde were similarly derivatized. Samples were analyzed by GC as described for the analysis of sinapoyl glucose.

Histochemistry

For histochemical examination of lignin in rachis segments, tissue was stained using the Mäule reagent. Hand sections of rachis segments

were fixed in 4% glutaraldehyde for 60 min, rinsed in water, and treated with 0.5% $KMnO_4$ for 10 min. Sections were rinsed with water, treated with 10% HCl for 5 min, rinsed with water, mounted in concentrated NH_4OH, and examined by bright-field microscopy.

Photography under UV Light

To record the appearance of thin-layer chromatograms as well as wild-type and *sin1* mutant plants under UV light, objects were photographed using a 365-nm transilluminator as a light source, Ektachrome EPY 404 film (Eastman Kodak, Rochester, NY), and a Schneider Kreuznach B&W #22 yellow filter (Schneider Kreuznach, Bad Kreuznach, Germany) to remove reflected UV light.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the gifts of authentic sinapic acid esters from Dr. Dieter Strack (Technische Universität, Braunschweig, Germany), the samples of ^{14}C-ferulate and ^{14}C-feruloyl coenzyme A contributed by Geoff Foxon (ICI Seeds, Jealott's Hill Research Station, Bracknell, Berks, United Kingdom), the careful review of the manuscript by Dr. Malcolm M. Campbell, and the excellent photographic work by Kurt Stepnitz. This work was supported in part by grants from the U.S. Department of Agriculture (No. 91-37304) and the U.S. Department of Energy (No. DE-FG02-90ER20021) (to C.R.S.), the Natural Sciences and Engineering Research Council of Canada (C.C.S.C. and B.E.E.), and the Alexander von Humboldt Foundation (T.V.). Mass spectral data were obtained at the Michigan State University Mass Spectrometry Facility, which is supported, in part, by a grant from the Biotechnology Research Technology Program, National Center for Research Resources, National Institutes of Health.

Received July 28, 1992; accepted September 18, 1992.

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NOTE ADDED IN PROOF

In view of the recent use of the locus designation *SIN1* in **Robinson-Beers et al.** (1992). Plant Cell **4,** 1237–1249, the sinapic acid biosynthesis mutant described in this paper will be renamed when a suitable, nonconflicting locus designation can be found.

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