

The *Arabidopsis ref2* Mutant Is Defective in the Gene Encoding CYP83A1 and Shows Both Phenylpropanoid and Glucosinolate Phenotypes

Matthew R. Hemm, Max O. Ruegger,¹ and Clint Chapple²

Department of Biochemistry, Purdue University, 175 South University Street, West Lafayette, Indiana 47907

The *Arabidopsis ref2* mutant was identified in a screen for plants having altered fluorescence under UV light. Characterization of the *ref2* mutants showed that they contained reduced levels of a number of phenylpropanoid pathway-derived products: sinapoylmalate in leaves, sinapoylcholine in seeds, and syringyl lignin in stems. Surprisingly, positional cloning of the *REF2* locus revealed that it encodes CYP83A1, a cytochrome P450 sharing a high degree of similarity to CYP83B1, an enzyme involved in glucosinolate biosynthesis. Upon further investigation, *ref2* mutants were found to have reduced levels of all aliphatic glucosinolates and increased levels of indole-derived glucosinolates in their leaves. These results show that CYP83A1 is involved in the biosynthesis of both short-chain and long-chain aliphatic glucosinolates and suggest a novel metabolic link between glucosinolate biosynthesis, a secondary biosynthetic pathway found only in plants in the order Capparales, and phenylpropanoid metabolism, a pathway found in all plants and considered essential to the survival of terrestrial plant species.

INTRODUCTION

The phenylpropanoid pathway converts Phe to a number of important secondary metabolites, including flavonoids, lignans, and lignin and hydroxycinnamic acid esters. Although the phenylpropanoid pathway is ubiquitous in vascular plants (Lewis and Yamamoto, 1990), downstream branches that lead to different classes of secondary metabolites often are unique to certain taxa, resulting in species-specific phenylpropanoid-derived products. Examples of these metabolites include the isoflavonoids found in members of the Fabaceae (Tahara and Ibrahim, 1995) and the sinapate esters found in members of the Brassicaceae (Strack, 1977; Chapple et al., 1992).

Sinapoylmalate and sinapoylcholine are two of the major sinapate esters of *Arabidopsis* (Chapple et al., 1992). Sinapoylmalate accumulates in leaves, where it acts as a UV light protectant (Landry et al., 1995; Booiij-James et al., 2000), whereas sinapoylcholine accumulates in embryos and is thought to serve as a storage form of choline and sinapic acid for germinating seedlings (Strack, 1981; Ruegger

et al., 1999). Under UV illumination, sinapoylmalate in the epidermis of wild-type plants causes their leaves to appear blue-green. By contrast, leaves of the sinapoylmalate-deficient *fah1* mutant appear red under UV light as a result of the fluorescence of chlorophyll (Chapple et al., 1992). The fluorescent property of sinapate esters makes them convenient visible markers for the genetic dissection of phenylpropanoid metabolism (Chapple et al., 1992; Ruegger and Chapple, 2001). The cloning and characterization of genes defective in these mutants has improved our understanding of the functions of known phenylpropanoid enzymes (Humphreys et al., 1999; Franke et al., 2002a, 2002b) and identified a new class of enzymes not previously known to be involved in secondary metabolism (Lehfeldt et al., 2000; Shirley et al., 2001).

In contrast to phenylpropanoid metabolism, the glucosinolate biosynthetic pathway generally is restricted to members of the order Capparales (Halkier and Du, 1997). Glucosinolates are a heterogeneous family of compounds derived from a variety of protein and nonprotein amino acids. Similar biosynthetic mechanisms convert these amino acids to secondary metabolites that share a common glucosinolate backbone but differ in an R group derived from the original amino acid precursor (Chapple et al., 1994). These compounds are substrates for the enzyme myrosinase, which degrades them into biologically active products thought to play roles in the defense against herbivory (Halkier and Du, 1997; Lambrix et al., 2001).

¹ Current address: Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268.

² To whom correspondence should be addressed. E-mail chapple@purdue.edu; fax 765-496-7213.

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Arabidopsis plants accumulate Met-, Phe-, and Trp-derived glucosinolates (Petersen et al., 2002). Despite decades of research, many gaps still exist in our understanding of the catalysts involved in glucosinolate biosynthesis (Bennett et al., 1995; Halkier and Du, 1997); however, the recent isolation and characterization of Arabidopsis glucosinolate mutants such as *sur2/rnt1* and *bus1/sps*, plants reduced in their content of Trp- and short-chain Met-derived glucosinolates, respectively, has led to significant advances in our understanding of this metabolic pathway (Barlier et al., 2000; Bak et al., 2001; Chen and Andreassan, 2001; Reintanz et al., 2001; Tantikanjana et al., 2001). Many of these mutants show morphological and developmental defects, and there is increasing evidence that alterations in glucosinolate biosynthesis can lead to changes in plant hormone levels (Barlier et al., 2000; Bak and Feyereisen, 2001; Bak et al., 2001; Reintanz et al., 2001; Tantikanjana et al., 2001).

It is now known that a number of the reactions in glucosinolate biosynthesis are catalyzed by cytochrome P450-dependent monooxygenases (P450s). Members of the CYP79 family of P450s are responsible for the initial step in glucosinolate biosynthesis, in which the amino acid precursor is oxidized to its respective aldoxime. The CYP79B2 and CYP79B3 enzymes oxidize Trp to indole 3-acetaldoxime (Hull et al., 2000; Mikkelsen et al., 2000); whereas CYP79F1 oxidizes short-chain Met-derived homologs (Hansen et al., 2001; Reintanz et al., 2001). Recent work with the *sur2* Arabidopsis mutants has shown that the next step in indole-derived glucosinolate biosynthesis is catalyzed by the cytochrome P450 CYP83B1 (Bak and Feyereisen, 2001; Bak et al., 2001; Hansen et al., 2001). These mutants accumulate less indole-derived glucosinolates than the wild type, and heterologously expressed CYP83B1 converts indole 3-acetaldoxime to a thiohydroximate adduct in the presence of a thiol donor (Bak and Feyereisen, 2001; Bak et al., 2001). It is becoming clear that the indole 3-acetaldoxime also is a precursor for indole 3-acetic acid, and as a result of defects in CYP83B1 activity, *sur2* shows phenotypes thought to be associated with auxin overaccumulation (Delarue et al., 1998; Barlier et al., 2000; Bak et al., 2001).

A recent screen of ~100,000 M2 seedlings for plants showing reduced fluorescence under UV light yielded multiple independent mutants disrupted in phenylpropanoid biosynthesis (Ruegger and Chapple, 2001). For all of these mutants, the reduced leaf fluorescence was found to be the result of significantly decreased levels of sinapoylmalate. Here, we report the further characterization of the *ref2* mutant of Arabidopsis and the isolation of the *REF2* gene. Our results show that *REF2* encodes CYP83A1, an enzyme suggested previously to be involved in the synthesis of non-Trp-derived glucosinolates (Bak and Feyereisen, 2001). Our work provides genetic evidence of the role of CYP83A1 in alkylglucosinolate biosynthesis and suggests that a block in alkylglucosinolate biosynthesis simultaneously perturbs phenylpropanoid metabolism in the *ref2* mutant.

RESULTS

REF2 Maps to BAC F18A5

To understand how mutations in the *REF2* gene affect phenylpropanoid biosynthesis, *REF2* was isolated by positional cloning. Using 20 F2 plants from a *ref2-1* (Columbia ecotype) × Landsberg *erecta* cross, a set of Arabidopsis restriction fragment length polymorphism markers spanning the Arabidopsis genome (Fabri and Schaffner, 1994; Schaffner, 1996) was used to identify an initial map position for the gene near the middle of chromosome 4. The position of *REF2* was delineated further using PCR-based codominant cleaved amplified polymorphic sequence markers (Konieczny and Ausubel, 1993) and simple sequence length polymorphism markers (Bell and Ecker, 1994) available on the TAIR World Wide Web site (www.arabidopsis.org). Sequence information from both the Cereon database (www.arabidopsis.org/Cereon/index.html; Cereon Genomics, Cambridge, MA) as well as the Landsberg *erecta* database (www.tigr.org/tdb/e2k1/ath1/atgenome/Ler.shtml) was used to generate additional cleaved amplified polymorphic sequence markers to screen a mapping population of 850 plants, eventually narrowing the mapping interval to a 60-kb region on BAC F18A5. According to these results, *REF2* is located between marker Cer466125 (31 kb from the centromeric end of BAC F18A5) and marker Cer466224 (89 kb) (Figure 1A).

REF2 Encodes CYP83A1

To identify the location of *REF2* on BAC F18A5, an overlapping contig was assembled from cosmid clones containing 6- to 14-kb fragments of Landsberg *erecta* genomic DNA. The clones were isolated by probing a Landsberg *erecta* genomic library constructed in the binary vector pBIC20 (Meyer et al., 1996) with PCR products of genomic DNA from the mapping interval. Thirty-five cosmid clones were isolated, with a minimum overlap of 10 kb between contiguous fragments. Using the floral dip method of Agrobacterium-mediated plant transformation (Clough and Bent, 1998), these binary cosmids were transformed into *ref2-1* plants. All kanamycin-resistant T1 progeny were screened visually and by HPLC analysis for complementation of the *ref2* phenotype. Four cosmids were found that complemented the *ref2* sinapoylmalate deficiency phenotype (Figure 2A). The minimum sequence overlap of these fragments spanned a 7.4-kb region of F18A5 that contained one annotated gene, the P450 *CYP83A1*, a gene cloned previously based on a slight homology with ferulate 5-hydroxylase (Chapple, 1995).

To examine the possibility that *REF2* is an unannotated gene in the 7.4-kb region of complementing DNA rather than *CYP83A1*, the steady state level of *CYP83A1* mRNA in the four *ref2* mutant alleles was assayed by RNA gel blot hybridization. RNA isolated from 4-week-old *ref2* and wild-type ro-

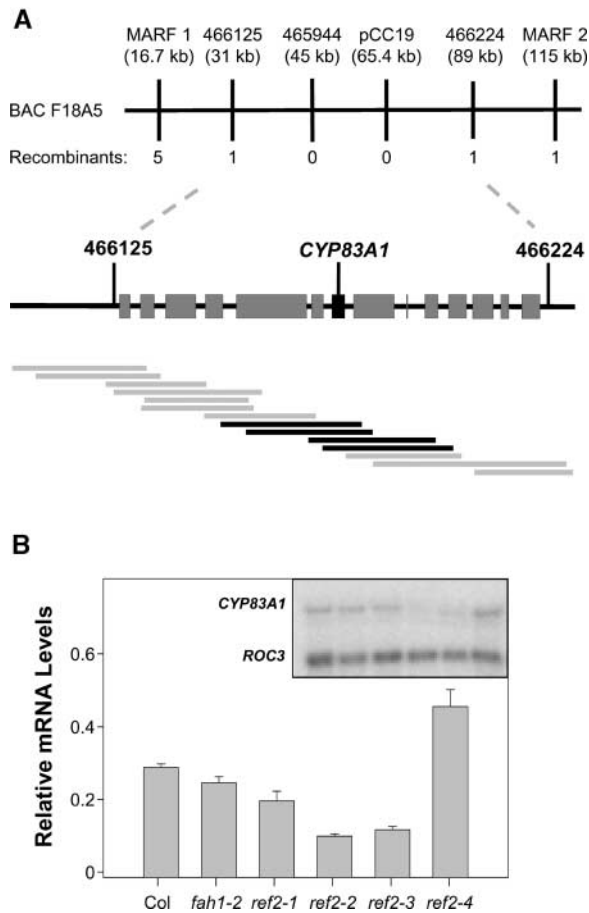


Figure 1. Map-Based Cloning of the *REF2* Gene.

(A) *REF2* was mapped to a 60-kb mapping interval on BAC F18A5 containing 14 annotated genes. A contig of cosmids containing Landsberg genomic DNA was constructed that spanned the mapping interval (gray bars). Cosmids that complemented the sinapoyl-malate and leaf glucosinolate phenotypes of *ref2-1* overlap at *CYP83A1* (black bars).

(B) RNA gel blot analysis of *CYP83A1* expression in wild-type and *ref2* rosettes. The graph shows levels of *CYP83A1* expression normalized to cyclophilin (*ROC3*) expression. Error bars represent standard errors of triplicate assays. Col, Columbia wild type.

rosettes was probed with a *CYP83A1* cDNA using a cyclophilin (*ROC3*) cDNA as a control (Chow and Gasser, 1997). The steady state level of *CYP83A1* expression was decreased substantially in three of the four *ref2* alleles (Figure 1B). Given that transcript levels of alleles containing frameshift or nonsense mutations frequently are decreased (Hillgren and Parker, 1999), these results strongly support the identity of *REF2* as *CYP83A1*.

As a final confirmation of the identity of *REF2*, the genomic DNA corresponding to all four mutant alleles was se-

quenced. Each *CYP83A1* allele from the *ref2* mutants was found to contain a unique G-to-A base change. In *ref2-1* and *ref2-3* alleles, the codons for Trp-58 and Trp-406, respectively, were converted to stop codons. In *ref2-2*, the G-to-A change altered a 3' intron-exon boundary recognition sequence (aaaaGGAT to aaaaaGAT; Simpson et al., 1998) such that it probably shifts the 3' intron-exon border downstream by one base, causing a frameshift and termination of the polypeptide after another two amino acids. Finally, in the protein encoded by the *ref2-4* allele, a conserved Gly in the proposed heme binding site at position 424 of the protein (Chapple, 1998) is converted to Glu. All four *ref2* mutations would be expected to strongly reduce or eliminate *CYP83A1* function (Graham and Peterson, 1999; Hillgren and Parker, 1999). These results confirm that *REF2* is *CYP83A1*.

The *ref2* Mutant Is Defective in Glucosinolate Biosynthesis

CYP83A1 shares 65% amino acid sequence identity with *CYP83B1* (Bilodeau et al., 1999), a cytochrome P450 that catalyzes an early reaction in the indole glucosinolate biosynthetic pathway (Bak and Feyereisen, 2001). The proposed reaction catalyzed by *CYP83B1* is the conversion of indole-3-acetaldoxime to 1-aci-nitro-2-indolyl-ethane, which reacts nonenzymatically to become an *S*-alkylthiohydroximate in the presence of a thiol donor (Bak et al., 2001; Hansen et al., 2001). To test the hypothesis that *CYP83A1* also acts in glucosinolate biosynthesis, desulfoglucosinolate extracts were prepared from wild-type and *ref2* tissues and analyzed by HPLC and liquid chromatography-mass spectrometry (LC-MS). HPLC analysis showed that *ref2* leaf extracts contain less short-chain (3-methylsulfinylpropyl, 4-methylthiobutyl, and 4-methylsulfinylbutyl) and long-chain (5-methylsulfinylpropyl, 5-methylthiopropyl, 7-methylsulfinylheptyl, 7-methylthioheptyl, 8-methylsulfinyloctyl, and 8-methylthio-octyl) Met-derived glucosinolates (Figure 3). Concomitant with the decreases in alkylglucosinolates, *ref2* leaf extracts show increased levels of the indole glucosinolates indol-3-ylmethyl, 4-methoxyindol-3-ylmethyl, and 1-methoxyindol-3-ylmethyl glucosinolate (Figure 3). Because of the decrease in alkylglucosinolates and the increase in indole glucosinolates, the major glucosinolate accumulated in *ref2* leaves is indol-3-ylglucosinolate. By contrast, the major glucosinolate present in wild-type leaves is 4-methylsulfinylbutyl glucosinolate.

Comparable reductions in alkylglucosinolate profiles were observed when samples of *ref2* seeds were analyzed (Figure 4); however, no consistent increases in indole glucosinolates were seen. In addition to decreases in the glucosinolates listed above, seeds of the mutants also contained significantly less benzoyloxyalkyl glucosinolates (Figure 4). Because the glucosinolate core structure of benzoyloxyalkyl glucosinolates is synthesized from Met in Arabidopsis (Graser et al., 2001), a decrease in alkylglucosinolate precursors

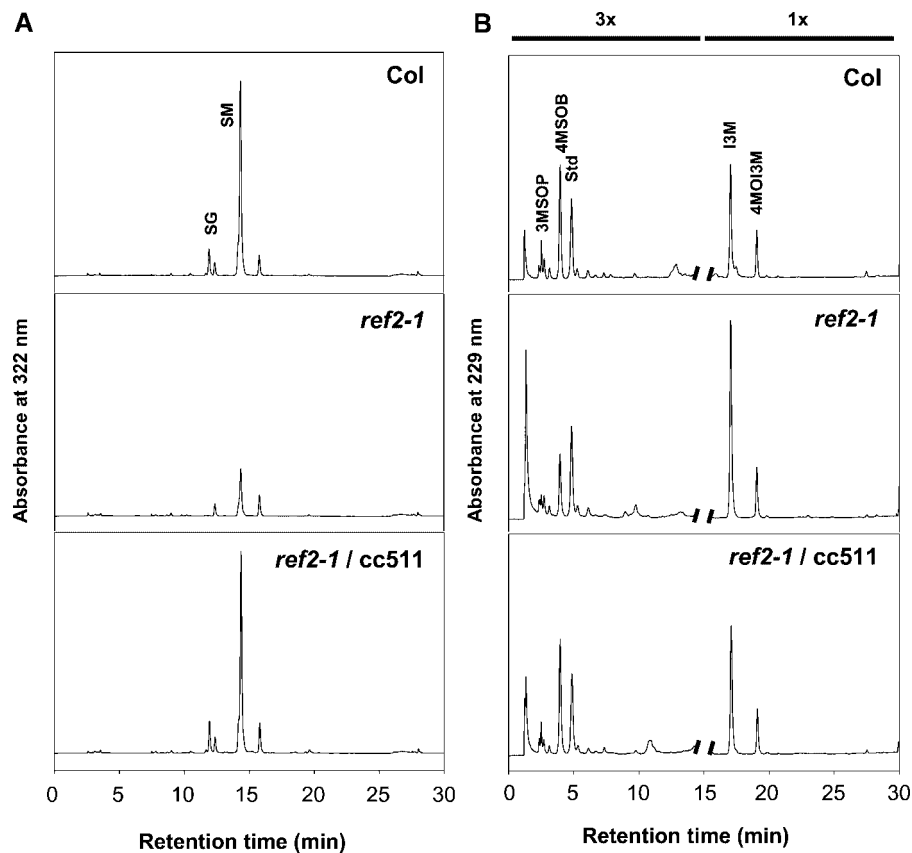


Figure 2. HPLC Analysis of Soluble Secondary Metabolites Accumulated in Leaves of Wild-Type, *ref2-1*, and a Representative *ref2-1* Plant Transformed with a Cosmid Containing *CYP83A1*.

(A) Methanolic leaf extracts analyzed by HPLC. The elution of UV light-absorbing compounds was monitored at 320 nm. Col, Columbia wild type; SG, sinapoylglucose; SM, sinapoylmalate.

(B) Leaf desulfoglucosinolate fractions analyzed by HPLC. The elution of UV light-absorbing compounds was monitored at 229 nm. Peaks are magnified threefold for the first 15 min of the chromatograms to more clearly show differences in peak areas. Glucosinolates are identified as follows: 3MSOP, 3-methylsulfinylpropyl; 4MSOB, 4-methylsulfinylbutyl; Std, internal standard; I3M, indol-3-ylmethyl; 4MOI3M, 4-methoxyindol-3-ylmethyl.

probably is responsible for the decreased levels of benzoylalkyl glucosinolates in *ref2* seeds. These results demonstrate that *CYP83A1* is required for the accumulation of wild-type levels of Met-derived glucosinolates in Arabidopsis (Figure 5).

As stated above, *REF2* was identified originally as *CYP83A1* based on the complementation of the *ref2-1* sinapoylmalate deficiency by cosmids containing the Landsberg *CYP83A1* genomic sequence. After determining that the *ref2* mutations affected glucosinolate accumulation as well, glucosinolate levels also were examined in *ref2-1* plants transformed with the *CYP83A1*-containing cosmids. HPLC analysis of desulfoglucosinolate extracts from leaves of wild-type, *ref2-1*, and transgenic *ref2-1* plants indicated

that the Landsberg *CYP83A1* sequence complemented the *ref2-1* alkylglucosinolate phenotypes as well as its phenylpropanoid defects (Figure 2B).

***ref2* Lacks the Morphological and Developmental Phenotypes Seen in Other Arabidopsis Glucosinolate Mutants**

Several previously identified Arabidopsis glucosinolate mutants show developmental abnormalities. These include *bus1*, which lacks wild-type levels of *CYP79F1* function and is blocked in the first step of short-chain alkylglucosinolate biosynthesis (Hansen et al., 2001; Reintanz et al., 2001), and

sur2, which eliminates CYP83B1 function and blocks the aldoxime-oxidizing reaction in indole glucosinolate biosynthesis (Barlier et al., 2000; Bak et al., 2001; Hansen et al., 2001). Other than a slight delay in average time to bolt (data not shown), analysis of a range of developmental characteristics

in *ref2* plants, including germination time and frequency, leaf size, internode length, and siliques shape, showed no obvious differences between wild-type and *ref2* plants. *ref2* inflorescences reached wild-type heights and displayed none of the obvious defects of *bus1* and *sur2* plants (Figure 6A).

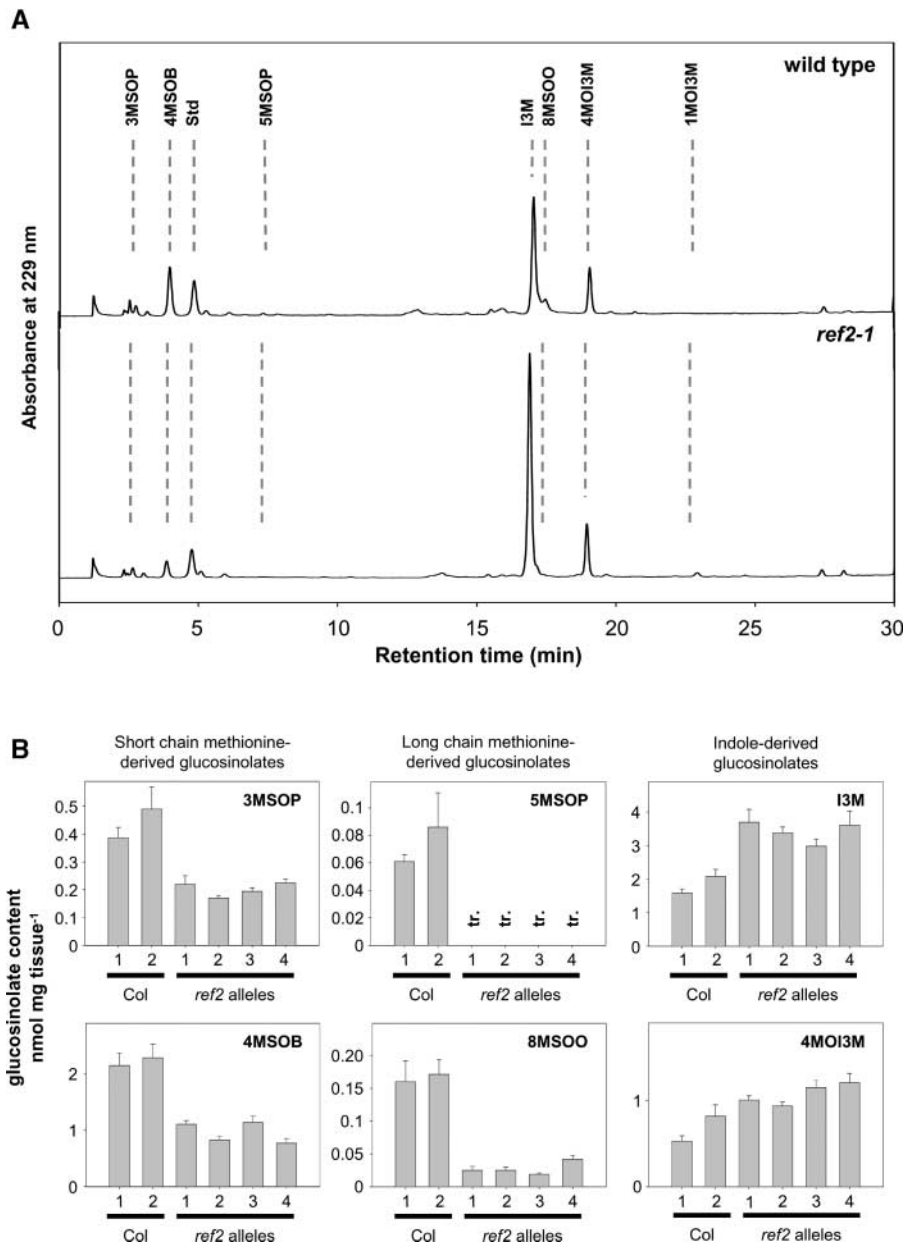


Figure 3. Analysis of Glucosinolate Levels in Wild-Type and *ref2* Leaves.

(A) Leaf desulfoglucosinolate fractions analyzed by HPLC. The elution of UV light-absorbing compounds was monitored at 229 nm. Glucosinolates are identified as follows: 3MSOP, 3-methylsulfinylpropyl; 4MSOB, 4-methylsulfinylbutyl; Std, internal standard; 5MSOP, 5-methylsulfinylpropyl; I3M, indol-3-ylmethyl; 8MSOO, 8-methylsulfinyloctyl; 4MOI3M, 4-methoxyindol-3-ylmethyl; 1MOI3M, 1-methoxyindol-3-ylmethyl.

(B) Quantification of the levels of representative glucosinolates in wild-type and *ref2* leaves. Error bars represent standard errors of at least triplicate assays. Col, Columbia wild type; tr., trace.

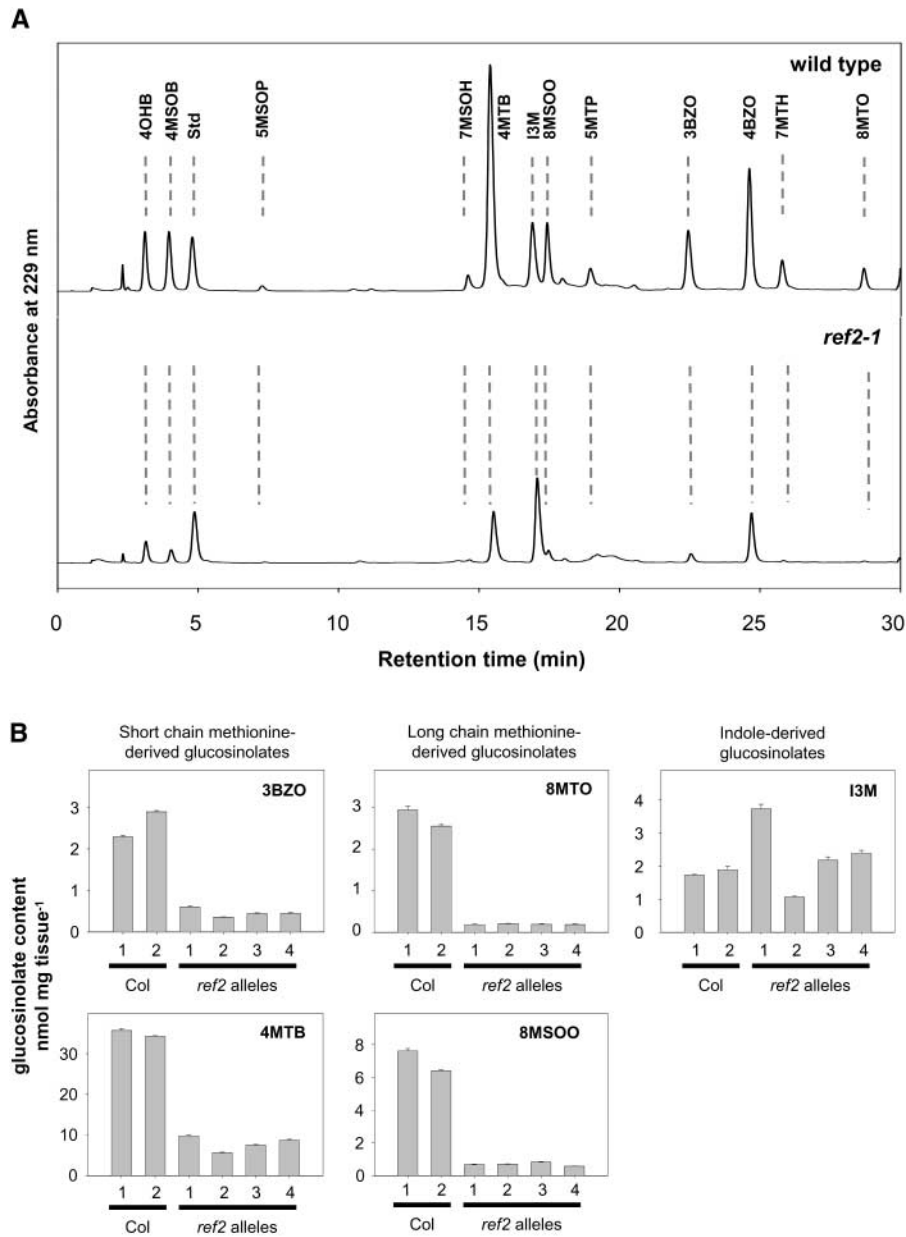


Figure 4. Analysis of Glucosinolate Levels in Wild-Type and *ref2* Seeds.

(A) Seed desulfoglucosinolate fractions analyzed by HPLC. The elution of UV light-absorbing compounds was monitored at 320 nm. Glucosinolates are identified as follows: 4OHB, 4-hydroxybutyl; 4MSOB, 4-methylsulfinylbutyl; Std, internal standard; 5MSOP, 5-methylsulfinylpropyl; 7MSOH, 7-methylsulfinylheptyl; 4MTB, 4-methylthiobutyl; I3M, indol-3-ylmethyl; 8MSOO, 8-methylsulfinyloctyl; 5MTP, 5-methylthiopropyl; 3BZO, 3-benzoyloxypropyl; 4BZO, 4-benzoyloxybutyl; 7MTH, 7-methylthioheptyl; 8MTO, 8-methylthiooctyl.

(B) Quantification of levels of representative glucosinolates in wild-type and *ref2* seeds. Error bars represent standard errors of at least triplicate assays. Col, Columbia wild type.

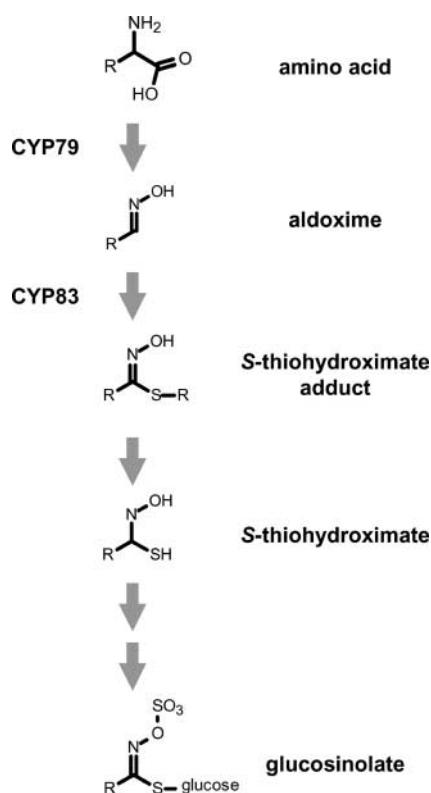


Figure 5. Scheme of Glucosinolate Biosynthesis.

The oxidation of amino acids to their respective aldoximes is catalyzed by cytochrome P450 monooxygenases in the CYP79 family. Members of the CYP83 family subsequently oxidize these aldoximes to a product that is thought to react spontaneously to give an S-thiohydroximate. These S-thiohydroximates are further glycosylated and sulfated to yield the final glucosinolates.

Similarly, light-grown *ref2* seedlings did not develop the adventitious roots seen in *sur2* plants (Figure 6C) as a result of auxin overproduction. Finally, dark-grown *ref2* seedlings exhibited a normal etiolated seedling phenotype compared with the reduced hypocotyl and root length phenotypes seen in *sur2* dark-grown seedlings (Figure 6B). These results show that a block at CYP83A1 in *ref2* plants does not lead to changes in hormone levels or metabolic pools capable of causing the phenotypes seen in related glucosinolate mutants.

Detailed Analysis Confirms the Lignin Phenotypes of *ref2*

A preliminary analysis of lignin composition had shown that *ref2* stems have wild-type levels of total lignin but that its lignin contains significantly reduced levels of syringyl mono-

mers (Ruegger and Chapple, 2001). Because the chemically resistant nature of lignin makes unambiguous analysis difficult, multiple methods of analysis often are used to achieve a more accurate evaluation of the lignin composition of plant tissue. Considering the unexpected role of REF2 in glucosinolate biosynthesis, the impact of the *ref2* mutation on lignin content was revisited using the Klason method (Kaar and Brink, 1991), and *ref2* lignin composition was assayed by the derivatization followed by reductive cleavage (DFRC) method (Lu and Ralph, 1997).

Klason analysis of *ref2* stems indicated that *ref2* plants accumulate wild-type levels of total lignin (Table 1). DFRC

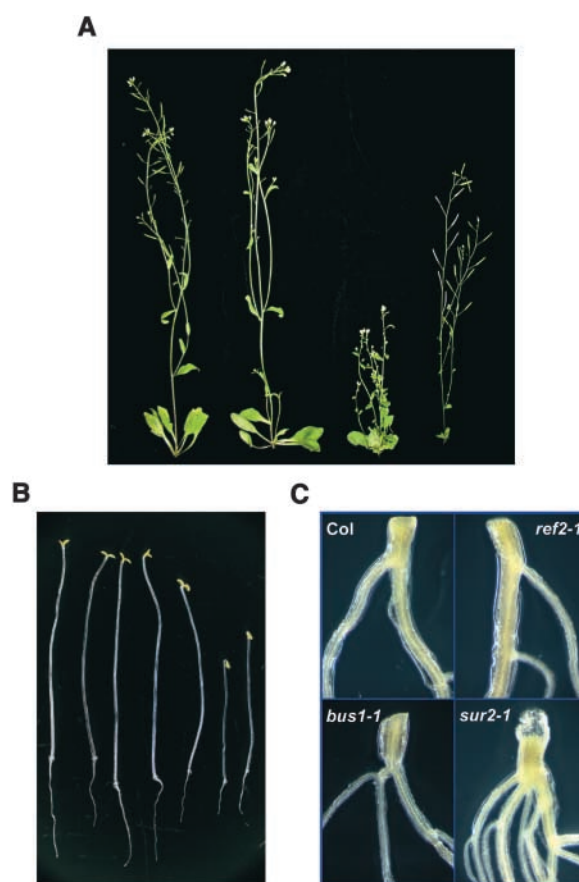


Figure 6. Morphology of *ref2* Plants Compared with Wild-Type Plants and with the Glucosinolate Biosynthetic Mutants *bus1* and *sur2*.

(A) Appearance of Columbia wild-type, *ref2-1*, *bus1*, and *sur2* plants (left to right).

(B) Appearance of 10-day-old etiolated seedlings of Columbia wild-type, *ref2-1*, *ref2-2*, *bus1*, Wassilewskija wild-type, and two seedlings of *sur2-1* (left to right).

(C) Hypocotyl-root junction of 2-week-old Columbia (Col) wild-type, *ref2-1*, *bus1*, and *sur2-1* light-grown seedlings.

Table 1. Klason Lignin Content and DFRC Lignin Monomer Composition of Mutant and Wild-Type Cell Walls ($n = 3$)

Sample	Klason lignin ($n = 3$) (mg/g cell wall)	Monomer Composition ($\mu\text{mol/g dry wt}$)			Monomer Composition ($\mu\text{mol/g Klason lignin}$)			Subunit mol %	
		G	S	Total	G	S	Total	G	S
Wild type	185 \pm 5	189 \pm 19	43 \pm 3	232 \pm 28	671 \pm 50	148 \pm 11	820 \pm 49	81 \pm 1	18 \pm 1
<i>ref2-1</i>	196 \pm 4	215 \pm 26	22 \pm 1	238 \pm 32	694 \pm 158	75 \pm 29	769 \pm 188	91 \pm 1	9 \pm 1
<i>ref2-2</i>	190 \pm 4	192 \pm 20	20 \pm 2	213 \pm 20	499 \pm 73	51 \pm 4	551 \pm 74	90 \pm 1	10 \pm 1
<i>ref2-3</i>	189 \pm 3	134 \pm 14	10 \pm 1	146 \pm 16	500 \pm 86	38 \pm 8	539 \pm 93	93 \pm 1	7 \pm 1
<i>ref2-4</i>	198 \pm 10	191 \pm 42	15 \pm 2	206 \pm 46	456 \pm 93	32 \pm 10	489 \pm 103	94 \pm 0.1	6 \pm 0.1

Values shown are for the units indicated \pm SE. G, guaiacyl; S, syringyl.

analysis of the same samples also confirmed the previous nitrobenzene oxidation results showing that the syringyl monomer content of *ref2* lignin was 25 to 50% of that found in wild-type lignin (Table 1). *ref2* cell walls contained, on average, 8% syringyl lignin, whereas wild-type cell walls contained \sim 18% syringyl lignin. Together, these assays suggest that the *ref2* mutation does not alter total carbon flux into the phenylpropanoid pathway but blocks phenylpropanoid biosynthesis downstream of guaiacyl lignin biosynthesis and upstream of sinapate ester-specific and syringyl lignin-specific reactions.

Perturbation of Phenylpropanoid O-Methylation Reactions May Be the Cause of the *ref2* Phenotype

Characterization of the *ref2* mutant's phenylpropanoid phenotypes strongly indicated some type of connection between alkylglucosinolate and phenylpropanoid biosynthesis, at least in the context of defective *ref2* alleles. Based on the current model of phenylpropanoid biosynthesis (Humphreys and Chapple, 2002), a block in either ferulate 5-hydroxylase (F5H) or caffeic acid O-methyltransferase (COMT) could lead to the *ref2* sinapate ester and syringyl lignin phenotypes. To investigate the possibility that the expression of these genes was reduced in the *ref2* background, steady state levels of F5H and COMT mRNA were assayed in 4-week-old *ref2* rosettes by RNA gel blot analysis (Figure 7). Quantification of expression levels using cyclophilin (*ROC3*) as a loading control showed that neither *F5H* nor *COMT* expression was decreased in *ref2* rosettes.

To determine if decreased F5H enzyme activity is responsible for the *ref2* phenylpropanoid phenotypes, attempts were made to assay F5H activity in plant microsomes prepared from wild-type and *ref2* rosettes. Unfortunately, activity could be detected in neither wild-type nor *ref2* microsomes, even using the enzyme's preferred substrates, coniferaldehyde and coniferyl alcohol (data not shown) (Humphreys et al., 1999; Osakabe et al., 1999). To circumvent the inability to assay F5H in vitro, a genetic approach was taken to investigate F5H activity in the *ref2* mutant. Although it has been stated frequently that *fah1* leaves lack

hydroxycinnamic acid esters, they accumulate a novel compound that is undetectable in wild-type extracts and that displays a UV light spectrum identical to that of ferulic acid (Figures 8A and 8B). LC-MS analysis of the peak showed that it has a mass consistent with that of feruloylmalate

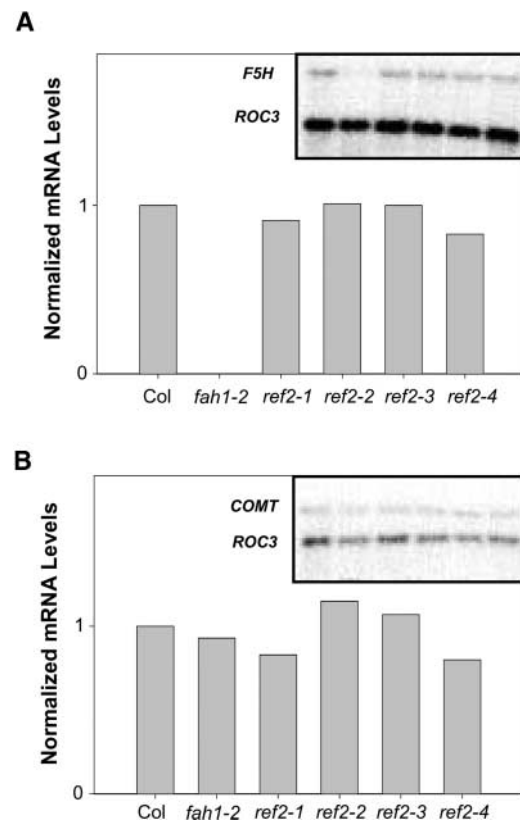


Figure 7. RNA Gel Blot Analysis of Phenylpropanoid Gene Expression in Wild-Type and *ref2* Rosettes.

(A) F5H gene expression in wild-type, *fah1-2*, and *ref2* rosettes normalized to cyclophilin (*ROC3*). Col, Columbia wild type.

(B) COMT gene expression in wild-type, *fah1-2*, and *ref2* rosettes normalized to cyclophilin (*ROC3*).

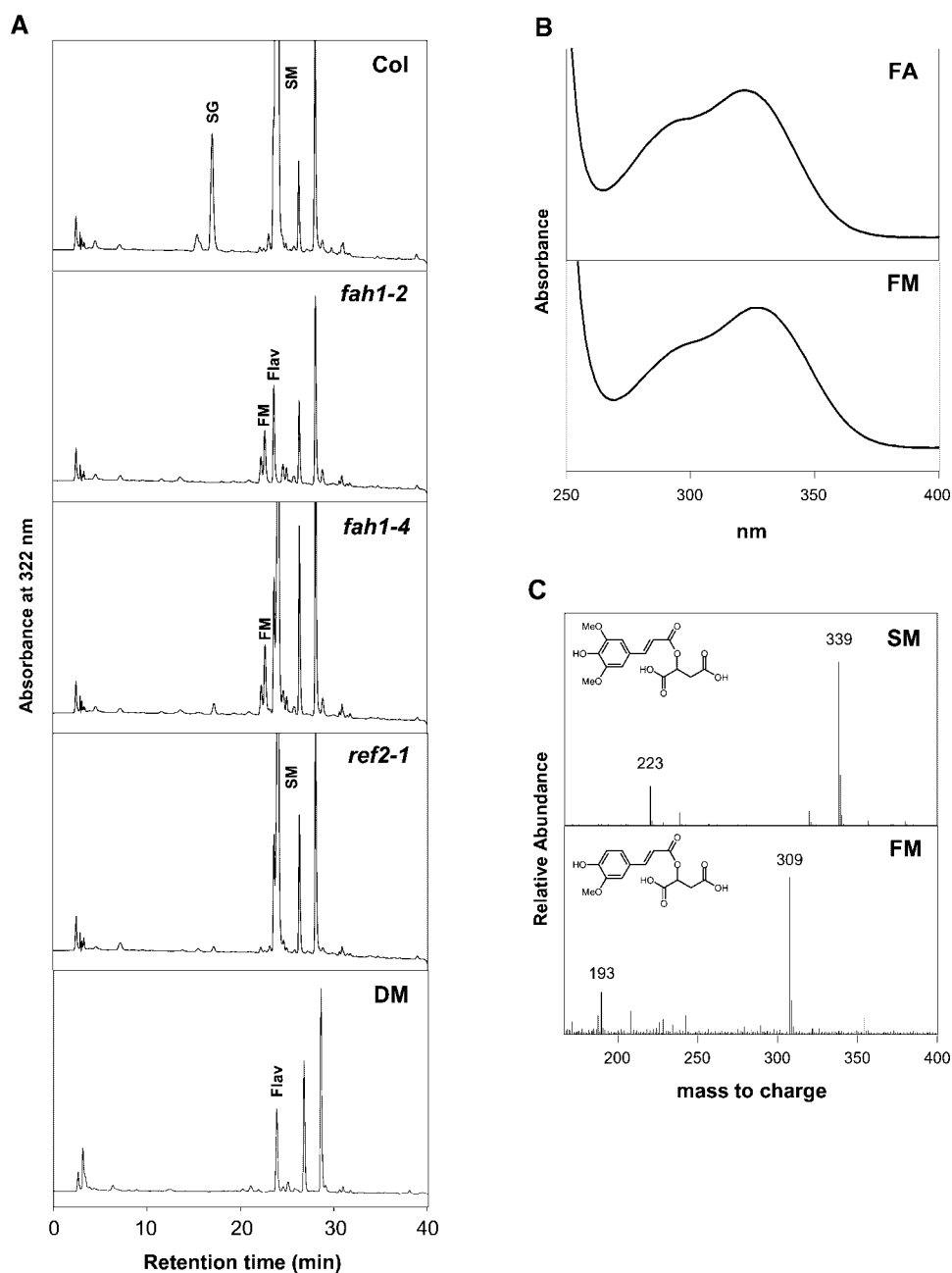


Figure 8. Soluble Secondary Metabolites Accumulated in the Leaves of the Wild Type, *fah1-2*, *fah1-4*, *ref2-1*, and a *fah1-2 ref2-1* Double Mutant.

(A) HPLC analysis of phenylpropanoids monitored at 320 nm. *fah1-2* is a null allele of *F5H*, whereas *fah1-4* is a leaky *fah1* allele. Col, Columbia wild type; DM, double mutant; Flav, flavonoid; FM, feruloylmalate; SG, sinapoylglucose; SM, sinapoylmalate.

(B) Absorbance spectrum of the novel ferulate ester peak accumulated in *fah1* mutants, and the spectrum of an authentic standard of ferulic acid (FA) for comparison.

(C) Mass spectra of sinapoylmalate from wild-type leaf extracts and feruloylmalate in *fah1-2* leaf extracts.

(negative ion mass-to-charge ratio of 309) (Figure 8C). This compound was detected in all null and leaky *fah1* alleles tested (Figure 8A) and was absent in wild-type extracts analyzed by HPLC and LC-MS. These findings suggest that feruloylmalate accumulation is a common phenotype shared by Arabidopsis mutants with decreased levels of F5H. Thus, as a measure of in vivo F5H activity, plants homozygous for each *ref2* allele were analyzed by HPLC for the presence of feruloylmalate; however, this compound was not found in any of the mutants (Figure 8A). The absence of detectable levels of feruloylmalate in *ref2* extracts suggests that *ref2* rosettes contain wild-type levels of F5H activity. Finally, to determine if a decrease in COMT activity was responsible for the *ref2* phenylpropanoid phenotypes, COMT activity was assayed in wild-type and *ref2* protein extracts. HPLC analysis of in vitro enzyme assays showed that *ref2* extracts have wild-type levels of COMT (Table 2).

If inhibitors of methylation reactions, including that catalyzed by COMT, accumulate in *ref2* leaves, in vitro analysis of COMT activity in desalted protein extracts in the presence of saturating levels of substrates may not accurately reflect the enzyme's in vivo activity. For this reason, and as a parallel to the genetic approach used to evaluate F5H action in vivo, the phenylpropanoid ester content of a *ref2-1 fah1-2* double mutant was evaluated. HPLC analysis of *ref2-1 fah1-2* leaf extracts showed that the double mutant lacked the sinapoylmalate peak present in *ref2-1* extracts and the feruloylmalate peak identified in *fah1-2* extracts (Figure 8A). Thus, *fah1* is epistatic to *ref2* for sinapoylmalate accumulation, whereas *ref2* is epistatic to *fah1* for the presence of feruloylmalate. These data reveal that at least in leaves, the *ref2* mutation can have an impact on phenylpropanoid metabolism upstream of F5H despite the fact that the *ref2* mutant deposits normal levels of guaiacyl lignin in stems. Given that the phenylpropanoid ring *O*-methylation steps catalyzed by caffeoyl-CoA *O*-methyltransferase and COMT are required for the synthesis of ferulic acid and sinapic acid, respectively, these two enzymes represent possible sites at which phenylpropanoid biosyn-

Table 2. COMT Activity and Ethylene Production in Wild-Type and *ref2* Arabidopsis ($n = 3$)

Sample	COMT Activity (pkat mg ⁻¹ protein)	Ethylene Production (ppb·mg ⁻¹ fresh wt·min ⁻¹)
Wild type	8.39 ± 0.78	9.4 ± 1.8
Wild type (2)	11.97 ± 0.33	–
<i>ref2-1</i>	9.37 ± 0.07	16.3 ± 1.4
<i>ref2-2</i>	8.25 ± 0.13	15.7 ± 3.0
<i>ref2-3</i>	10.46 ± 0.14	–
<i>ref2-4</i>	9.74 ± 0.36	–

Values shown are for the units indicated ± SE.

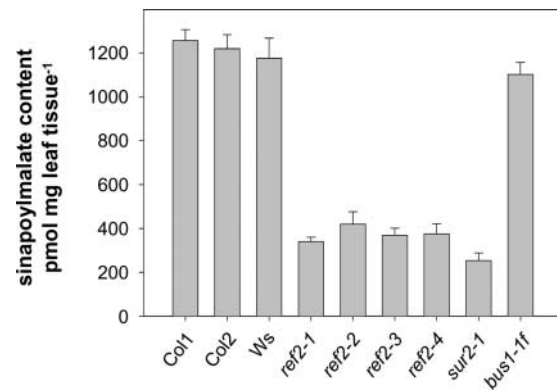


Figure 9. Sinapoylmalate Levels in Columbia Wild-Type, Wassilewskija Wild-Type, *ref2*, *sur2-1*, and *bus1* 4-Week-Old Rosettes.

Quantification of sinapate esters by HPLC was performed by comparison with a sinapate standard at 320 nm. Error bars represent standard errors of triplicate assays. Col, Columbia; Ws, Wassilewskija.

thesis could be perturbed in the *ref2* mutant. In this context, it is notable that *ref2* rosettes produce normal levels of ethylene (Table 2), suggesting that if phenylpropanoid *O*-methylation reactions are perturbed in the mutant, it is probably not the result of the general poisoning of *S*-adenosylmethionine pools.

sur2 Exhibits a *ref* Phenotype

To further investigate the link between glucosinolate and phenylpropanoid biosynthesis in the *ref2* mutant, the sinapate ester levels in leaves of two other glucosinolate biosynthetic mutants were analyzed by HPLC. Analysis of *bus1* leaf extracts showed that *bus1* has wild-type levels of leaf sinapate esters (Figure 9). Given that the *bus1* mutation blocks alkylglucosinolate biosynthesis immediately preceding the reaction catalyzed by REF2, this result suggests that the *ref2* phenylpropanoid phenotypes are not attributable to a lack of alkylglucosinolate intermediates downstream of both REF2 and BUS1 but instead probably are the result of an accumulation of the REF2 substrate, Met-derived aldoximes.

HPLC analysis of *sur2* leaf extracts showed that they contain significantly less sinapoylmalate than Wassilewskija, the parent ecotype of *sur2-1* (Figure 9). The reduced sinapate ester content of *sur2* rosettes shows that mutations in other glucosinolate biosynthetic genes lead to alterations in phenylpropanoid biosynthesis. Furthermore, given that CYP83B1 mutants may accumulate increased levels of indole-3-acetaldoxime, it also suggests that that perturbation of phenylpropanoid biosynthesis may be an effect common to multiple aldoximes.

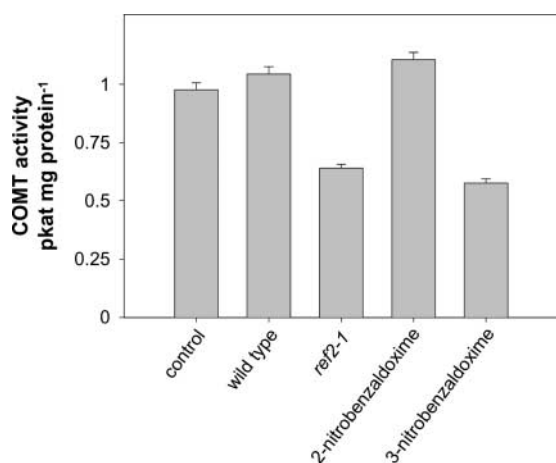


Figure 10. Activity of Heterologously Expressed COMT in the Presence of Wild-Type and Mutant Leaf Extracts and Synthetic Aldoximes.

Quantification of the ferulic acid product by HPLC was performed by comparison with a ferulic acid standard at 320 nm. Error bars represent standard errors for quadruplicate assays.

COMT Assayed in Vitro Is Inhibited by *ref2* Leaf Extracts and Synthetic Aldoximes

To further test the hypothesis that aldoximes accumulate in *ref2* tissues and inhibit phenylpropanoid *O*-methylation reactions, the activity of heterologously expressed COMT was assayed after preincubation with two commercially available aldoximes and with methanolic leaf extracts from wild-type and *ref2* plants (Figure 10). Based on the assumption that aldoximes may be competitive inhibitors of COMT, caffeic acid and *S*-adenosylmethionine were added to assays at concentrations close to the reported K_m values of COMT for these substrates (Edwards and Dixon, 1991). Compared with assays containing wild-type extracts, COMT activity by *ref2* extracts was between 25 and 38% in three independent experiments (Figure 10). These results indicate that *ref2* leaves may contain one or more inhibitors of COMT activity, including Met-derived aldoximes or their degradation products. Similarly, 3-nitrobenzaloxime, but not its 2-nitro isomer, inhibited COMT activity by 40%, further suggesting that aldoximes may play a role in the *ref2* phenylpropanoid phenotypes.

DISCUSSION

Genetic approaches can elucidate the functions of known proteins in plant metabolism but also have the potential to

identify novel factors that regulate or otherwise affect biosynthetic pathways. The *ref2* mutant was identified in a screen for plants showing altered phenylpropanoid phenotypes, but further analysis of *ref2* showed that mutations in *CYP83A1* also lead to decreased levels of glucosinolates, a family of secondary compounds thought to be metabolized independently from phenylpropanoids. With this fact in mind, a more detailed investigation into the phenylpropanoid, glucosinolate, and morphological phenotypes of *ref2* was conducted to determine if these phenotypes were the result of changes in phenylpropanoid pathway gene expression, a specific metabolic interaction between these two pathways, or a broad metabolic disruption in the mutant.

CYP83A1 Is an Enzyme in Met-Derived Glucosinolate Biosynthesis

Analysis of glucosinolates in *ref2* leaf and seed extracts shows that *ref2* plants accumulate significantly lower amounts of all of the alkylglucosinolates found in wild-type plants. Given that heterologously expressed CYP83A1 has been shown to be an aldoxime-oxidizing enzyme (Bak and Feyereisen, 2001), this genetic analysis shows that in vivo, CYP83A1 oxidizes Met-derived aldoximes in alkylglucosinolate biosynthesis (Figures 5 and 11). These results are in contrast to similar analyses conducted with *bus1* tissues, which showed that the *bus1* mutation leads to reductions in only short-chain Met-derived glucosinolates. Thus, although the oxidation of chain-elongated Met homologs is shared in a nonredundant manner by CYP79F1 and CYP79F2, CYP83A1 oxidizes all Met-derived aldoximes.

There May Be Distinct Functions for, but Compensatory Interplay between, CYP83A1 and CYP83B1

Residual levels of alkylglucosinolates in the four null *ref2* alleles examined in this study indicate that one or more other enzymes, most likely CYP83B1, can oxidize Met-derived aldoximes in the absence of CYP83A1 function (Figure 11). Similarly, indole glucosinolate levels are decreased by only ~50% in a null *sur2* allele (Bak et al., 2001). These data could be interpreted as evidence to support overlapping roles for CYP83A1 and CYP83B1 in glucosinolate biosynthesis, particularly in light of the recent demonstration that these enzymes oxidize a range of aldoximes (Bak and Feyereisen, 2001). By contrast, the accumulation of wild-type or greater levels of indole glucosinolates in *ref2* argues against a significant role for CYP83A1 in their biosynthesis, at least in the presence of wild-type CYP83B1 function. The lack of *sur2*-like developmental phenotypes in *ref2* plants also is consistent with this hypothesis, because a loss of CYP83A1 activity did not lead to indole 3-acetic acid-related growth perturbations. Together, these results

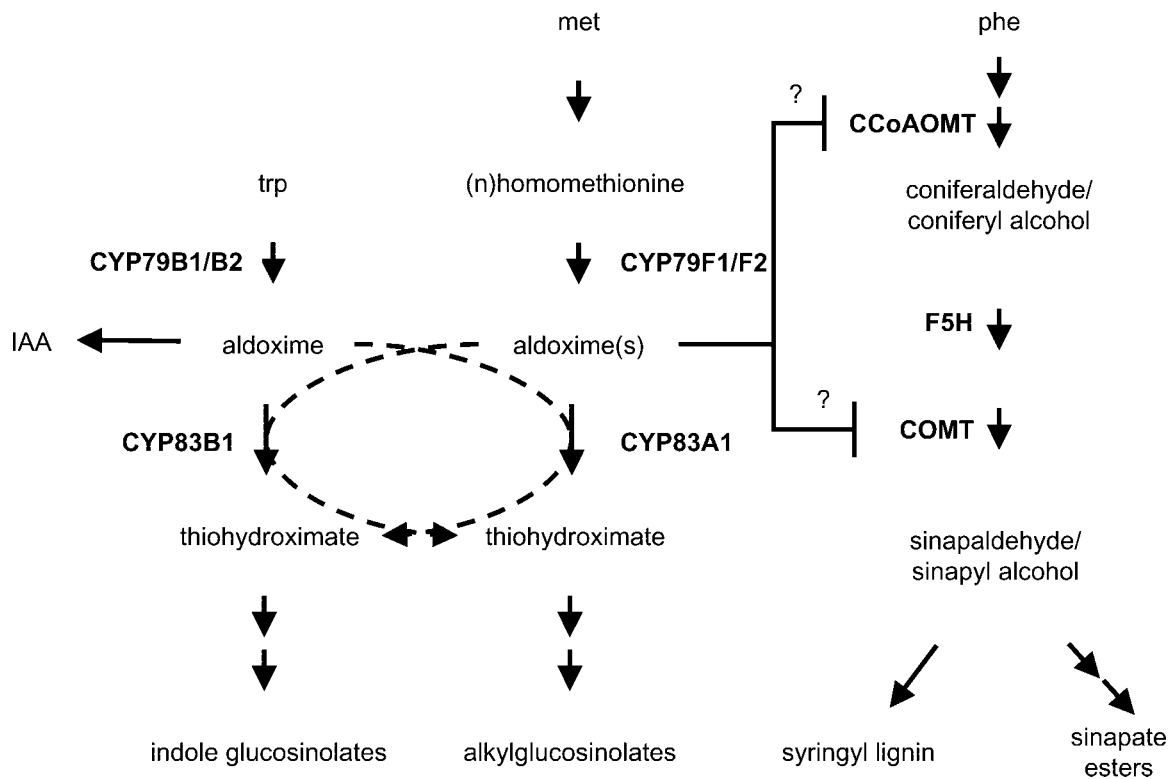


Figure 11. Proposed Model for Alkylglucosinolate and Phenylpropanoid Biosynthesis and Their Interaction in *ref2*.

The model illustrates the role of the six P450s known to function in alkylglucosinolate and indole glucosinolate biosynthesis. The model also indicates that CYP83A1 and CYP83B1 may have partially overlapping functions, at least in the context of certain mutants. Finally, the model illustrates the hypothesis that a block at CYP83A1 may lead directly or indirectly to decreased COMT and caffeoyl-CoA *O*-methyltransferase (CCoAOMT) activity as a result of the accumulation of Met-derived aldoximes. IAA, indole 3-acetic acid.

suggest that in wild-type plants, the primary functions of CYP83A1 and CYP83B1 are in alkylglucosinolate and indole glucosinolate biosynthesis, respectively; however, in the *sur2* and *ref2* mutants, the loss of one enzyme is mitigated by the activity of its homolog.

Interestingly, *ref2* leaf extracts were found consistently to contain higher levels of indole glucosinolates, a phenotype shared by *bus1* plants (Reintanz et al., 2001). These observations indicate that a block in alkylglucosinolate biosynthesis, regardless of whether it is at the amino acid- or the aldoxime-oxidizing step, leads to an increase in indole glucosinolates. Similarly, 3-methylsulfinylpropyl glucosinolate, the most abundant glucosinolate in Wassilewskija leaves, is hyperaccumulated in *sur2* (M. Hemm and C. Chapple, unpublished results). These results suggest that glucosinolate biosynthesis may be regulated in *Arabidopsis* such that a block in the biosynthesis of one class of glucosinolates leads to an increase in the synthesis of others, resulting in some degree of glucosinolate homeostasis.

Phenylpropanoid Biosynthesis Is Altered in *ref2*

Given that *ref2* mutants are morphologically indistinguishable from wild-type plants, it seems unlikely that their phenylpropanoid phenotypes are caused by broad-scale perturbation of hormone levels or plant development. Instead, biochemical analysis of *ref2*, and the inhibition of COMT by *ref2* extracts in vitro, suggest that the phenylpropanoid phenotypes of the mutant may be attributable to the inhibition of *O*-methyltransferase activities by one or more aldoximes accumulated in the mutant (Figure 11). Furthermore, the *ref* phenotype displayed by *sur2* plants, and the inhibition of COMT by 3-nitrobenzaldoxime, suggest that the perturbation of phenylpropanoid metabolism may result from the accumulation of a wide variety of aldoximes. Alternatively, because aldoximes are relatively reactive compounds (Dawson et al., 1993), it might be their degradation products that inhibit COMT.

Whether the inhibition of *O*-methylation reactions alone leads to the *ref2* phenylpropanoid phenotypes remains to be

determined. Another possible explanation for the *ref2* phenylpropanoid phenotypes is that CYP83A1 plays an enzymatic role in sinapate ester and/or syringyl lignin biosynthesis. Although it is difficult to explain where another P450 could act in the phenylpropanoid pathway, two other members of the CYP83 P450 family, CYP83C1 (Bilodeau et al., 1999) and CYP83D1 (Siminszky et al., 1999), have been identified in parsley and soybean, respectively, two species that do not accumulate glucosinolates. The presence of CYP83 family members in these taxa suggest that these enzymes may be involved in pathways other than glucosinolate biosynthesis, potentially in phenylpropanoid metabolism.

The Impact of *ref2* Mutations on Phenylpropanoid Metabolism May Constrain Glucosinolate Variability in Arabidopsis Populations

Recently, areas of the Arabidopsis genome have been identified as quantitative trait loci for glucosinolate accumulation. For example, quantitative trait loci have been identified that lead to differences in the levels of glucosinolates between the Landsberg and Cape Verdi ecotypes (Kliebenstein et al., 2001). Neither *BUS1* nor *SUR2* is located in significant quantitative trait loci, consistent with the selective disadvantage that mutations at these loci would provide in natural populations because of their impact on overall plant vigor (Delarue et al., 1998; Bak et al., 2001; Reintanz et al., 2001). By contrast, *REF2* is contained within one of the quantitative trait loci responsible for a significant increase in indole glucosinolates in the Landsberg ecotype, suggesting that allelic variation at the *REF2* locus may exist among Arabidopsis ecotypes. In this context, however, it is interesting that plants lacking wild-type levels of sinapoylmalate show increased susceptibility to damage from UV light (Landry et al., 1995; Booij-James et al., 2000). Thus, even though null *CYP83A1* mutants develop normally under laboratory conditions, changes in glucosinolate biosynthesis through allelic variation at the *REF2* locus may be constrained in natural populations by the effects of decreased CYP83A1 activity on the synthesis of UV light-protective sinapate esters. This illustrates the potential for natural variation in one group of secondary metabolites to be linked directly to the accumulation of a group of biochemically unrelated molecules and for selection for variation in metabolites involved in plant-insect interactions to be constrained by relative plant fitness in the context of UV light stress.

METHODS

Plant Growth and Genetic Mapping

Arabidopsis thaliana plants were cultivated at a light intensity of 100 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at 23°C under a 16-h-light/8-h-dark photoperiod in Redi-

Earth potting mix (Scotts-Sierra Horticulture Products, Marysville, OH). Unless noted otherwise, all Arabidopsis plants used in the experiments were ecotype Columbia. As described previously, the four *ref2* alleles were isolated from a F2 screen of ethyl methanesulfonate-mutagenized plants (Ruegger and Chapple, 2001). *ref2-1* and *ref2-2* were backcrossed three times, and the *ref2-3* and *ref2-4* alleles were backcrossed once to Columbia before analysis. Isolation and backcross information on the *sur2-1* and *bus1-1* mutants has been described elsewhere (Delarue et al., 1998; Reintanz et al., 2001).

Cloning and Plant Transformation

The *ref2* mutant (Columbia background) was used in a cross to the Landsberg *erecta* ecotype. F1 individuals were allowed to self-pollinate, and F2 plants were screened for the *ref2* phenotype. DNA was extracted from homozygous mutant lines for Arabidopsis restriction fragment length polymorphism marker mapping (Schaffner, 1996) to determine an initial map position for the *REF2* gene. Subsequently, DNA was extracted from additional F2 plants and F3 families for use in PCR-based genotyping experiments. Individuals carrying recombinant chromosomes in the region of the *REF2* locus were used to determine a mapping interval for the gene and were analyzed further.

Cosmids containing Landsberg genomic fragments spanning the *REF2* locus were identified from a transformation-competent Landsberg genomic library (Meyer et al., 1996). These binary cosmid vectors then were introduced into *Agrobacterium tumefaciens* C58 pGV3850 (Zambrisky et al., 1983) by electroporation. Plant transformation was performed by the floral dip method (Clough and Bent, 1998).

To screen for transformants, *ref2-1* T0 seeds were surface-sterilized for 10 min in a 2:1 mixture of 0.1% Triton X-100 and bleach. Seeds were rinsed thoroughly with sterile water and plated on modified Murashige and Skoog (1962) medium (ammonia-free medium to which an additional 20.6 mM potassium nitrate was added in place of ammonium nitrate) containing 1% Suc, 0.7% agar, 50 mg/L kanamycin, and 200 mg/L Timentin. After 2 weeks, kanamycin-resistant progeny were transferred to soil. Two to 3 weeks after transplanting, plants were screened visually and by HPLC analysis for complementation of the *ref2* fluorescence and sinapoylmalate phenotypes.

RNA Gel Blot Analysis

RNA was isolated from 4-week old Arabidopsis rosettes as described previously (Goldsbrough and Cullis, 1981). RNA samples were separated electrophoretically, transferred to Hybond N⁺ membranes (Amersham), and hybridized at 65°C with a DNA probe (DECAprime II system; Amersham) made from PCR amplification of the gene of interest as well as with a probe made from the cDNA of the Arabidopsis cyclophilin *ROC3*. Gene expression was quantified using a Typhoon Imager (Molecular Dynamics, Sunnyvale, CA).

Glucosinolate Analysis

For glucosinolate analysis of plant tissues, desulfoglucosinolate extracts were prepared according to the protocol described by Glover et al. (1988) with minor modifications. Leaves were harvested and extracted twice in 1.5 mL of 50% methanol heated to 70°C for 15 min. Before heating, 40 μL of 500 μM allylglucosinolate (sinigrin) (Indofine Chemical Company, Somerville, NJ) was added to the first methanol extraction of each sample as an internal standard. Extracts were combined and dried

completely under vacuum using an RC1010 Speed-Vac (Jouan, Winchester, VA). Samples were redissolved in 1.5 mL of water and passed over an SP-50 anion-exchange column (500- μ L bed volume suspended in water). Columns then were washed with 3 mL of water. A total of 200 μ L of a 1:3 dilution of type-I sulfatase solution (*Helix pomatia*; Sigma) in 26 mM sodium acetate buffer, pH 5.5, was added to each column. Sulfatase was prepared as described by Kiddle et al. (2001). Columns were capped and incubated for at least 12 h at 37°C. After incubation, 1.5 mL of water was passed through the column and collected as the desulfoglucosinolate-containing fraction. For seed samples, the same protocol was used with the following alterations: 40 μ L of 5 mM allylglucosinolate was added to each sample as a standard, and samples weighing ~40 mg were incubated in 1 mL of 50% methanol for 10 min at 70°C to soften the seeds followed by grinding in the methanol and further incubation at 70°C for 20 min.

Desulfoglucosinolates were analyzed by diode array HPLC and liquid chromatography–mass spectrometry (LC-MS). A total of 100 μ L of the desulfoglucosinolate fractions was injected onto a Varian C18 reverse-phase column and run using the HPLC method described by Reintanz et al. (2001). Peaks were identified based on retention times from published results as well as on UV light spectra (Reintanz et al., 2001). Peak identity was confirmed by LC-MS (LCMS-2010 single-quadrupole mass spectrometer detector with an atmospheric pressure chemical ionization source; Shimadzu, Kyoto, Japan). The LCMS-2010 was operated under the following conditions: curved desolvation line temperature of 250°C, atmospheric pressure chemical ionization probe temperature of 400°C, nitrogen nebulizer gas flow of 2.5 L/min, detector voltage of 1.5 kV, and curved desolvation line voltage of –35 kV. Major peaks were identified by scanning ions with mass-to-charge ratios of 150 to 400 in positive mode. Quantification of glucosinolates was performed by comparison with the allylglucosinolate internal standard using HPLC response factors for desulfoglucosinolates at 229 nm (J. Gershenzon, personal communication).

Sinapate Ester Analysis

For analysis of sinapate esters, tissue was extracted in 50% methanol and analyzed by reverse-phase HPLC. Leaf and seed extracts were separated on a Microsorb-MV C18 column (Rainin Instruments, Woburn, MA) using a gradient from 1.5% acetic acid to 35% acetonitrile in 1.5% acetic acid at a flow rate of 1 mL/min. To better separate the novel ferulic acid ester found in *fah1-2* plants from the sinapoylmalate found in wild-type and *ref2* plants, samples were run on the same column using a gradient from 5% acetic acid to 25% acetonitrile in 5% acetic acid at a flow rate of 1 mL/min. Compounds were identified based on their retention times and UV light spectra. The identity of the feruloylmalate present in *fah1* leaf extracts was confirmed by LC-MS using the same system and method described for glucosinolate analysis. Samples were run on a Microsorb-MV C18 column using a gradient from 0 to 35% acetonitrile at a flow rate of 1 mL/min. Major peaks were identified by scanning ions with mass-to-charge ratios of 150 to 400 in negative and positive modes. Sinapate and ferulate esters were quantified based on comparison with standards of their respective free acids.

Lignin Analysis

For cell wall preparation, Arabidopsis rachis tissue was ground to a fine powder and extracted with neutral phosphate buffer, 80% etha-

nol, and acetone (Meyer et al., 1998). To measure lignin content, cell wall samples were analyzed using the microscale Klason method (Kaar and Brink, 1991). Lignin monomer composition was determined by the derivatization followed by reductive cleavage method (Lu and Ralph, 1997) modified as described previously (Franke et al., 2000). Derivatization followed by reductive cleavage products were quantified by comparison with authentic standards using gas chromatography–mass spectrometry and selective ion monitoring.

Ethylene Measurements

Rosettes of 4-week-old plants were harvested, weighed, and incubated under ambient laboratory lighting in a sealed 5-mL scintillation vial for 90 min. Ethylene content of the gas phase was measured as described previously (Jones and Woodson, 1999).

O-Methyltransferase Assays

Protein extracts were prepared and assayed for caffeic acid O-methyltransferase activity as described previously (Inoue et al., 1998) except that the product was measured by HPLC. Samples were separated on a Microsorb-MV C18 column (Rainin Instruments) using a gradient from 1.5% acetic acid to 35% acetonitrile in 1.5% acetic acid at a flow rate of 1 mL/min. Formation of the ferulic acid product was measured at 322 nm and quantified by comparison with standards. Total protein content was measured using the Pierce bicinchoninic acid assay using BSA as a standard.

Protein extracts from *Escherichia coli* expressing caffeic acid O-methyltransferase were prepared as described previously (Humphreys et al., 1999). Methanolic leaf extracts were prepared by incubating ground rosettes in 50% methanol for 15 min at 4°C. After samples were centrifuged, the supernatants were removed, stored at –70°C, and used in the add-back assays. In each assay, 10 μ L of plant extracts was incubated in a total volume of 100 μ L. Control assays contained 10 μ L of 50% methanol. In reactions incubated with 2-nitrobenzaldehyde or 3-nitrobenzaldehyde, 5 μ L of 10 mM aldehyde was incubated in a total volume of 100 μ L, making the final aldehyde concentration 500 μ M. Enzyme and extracts were incubated at 30°C for 30 min before reactions were started by the addition of S-adenosylmethionine and caffeic acid to final concentrations of 5 μ M S-adenosylmethionine and 60 μ M caffeic acid. After 1 h at 30°C, the reactions were stopped by the addition of 100 μ L of methanol followed by vortexing. Supernatants of the reaction were analyzed by HPLC.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

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The Arabidopsis ref2 Mutant Is Defective in the Gene Encoding CYP83A1 and Shows Both Phenylpropanoid and Glucosinolate Phenotypes

Matthew R. Hemm, Max O. Ruegger and Clint Chapple

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