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Biochemical Genetics of Plant Secondary Metabolites in *Arabidopsis thaliana*[1]

The Glucosinolates

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ABSTRACT

Mutants of *Arabidopsis thaliana* with a glucosinolate content different from wild type were isolated by screening a mutagenized population of plants. Six mutants were detected out of a population of 1200 screened. One of these mutants, TU1, was analyzed in detail. Leaf and seed tissues of line TU1 lack or have reduced amounts of many of the aliphatic glucosinolates found in the wild type due to a recessive allele, *gsm1*, of a single nuclear gene, *GSM1*. The seed phenotype is inherited as a maternal effect suggesting that the embryo is dependent on the maternal tissue for its glucosinolates. Experiments involving feeding of ^{14}C-labeled intermediates suggested that the *gsm1* allele results in a metabolic block which decreases the availability of several amino acid substrates required for glucosinolate biosynthesis: 2-amino-6-methylthiohexanoic acid, 2-amino-7-methylthioheptanoic acid, and 2-amino-8-methylthiooctanoic acid. The mutation does not result in any obvious changes in morphology or growth rate. A pathway for the biosynthesis of glucosinolates in *A. thaliana* is proposed.

Glucosinolates are anionic thioglucosides (Fig. 1) synthesized by many species of the order Capparales including all the Brassicaceae (for reviews see refs. 6 and 26). The primary biological function of glucosinolates is unknown, although a role in plant defense against bacterial and fungal pathogens and insect predators has been suggested (reviewed by Fenwick *et al.* [6]).

The presence of glucosinolates in crop species has several important consequences. First, dietary problems in livestock can result when fodder with high levels of glucosinolates are consumed. Thus, glucosinolates severely restrict the amount of glucosinolate-containing meal that can be used in animal feed supplements (24). Second, the distinctive flavor associated with Brassicaceae species which serve as vegetable and condiment crops (*e.g.* mustard, cabbage) is due, primarily, to isothiocyanates derived from the catabolism of glucosinolates. Finally, the isothiocyanates derived from the catabolism of many glucosinolates, are toxic to some bacterial and fungal pathogens. The same isothiocyanates discourage feeding by some insect species but act as attractants or behavior modifying chemicals for others. The possibility of improving the commercial value of some crops by manipulating the levels of glucosinolates in certain tissues has generated interest in understanding the biochemistry and genetics of glucosinolate biosynthesis.

Much of our knowledge concerning the biosynthesis of glucosinolates is derived from *in vivo* labeled precursor studies. These studies led to the proposal of a general glucosinolate biosynthetic pathway (17, 26). In essence, amino acids are considered to be the precursors of all glucosinolates. Both protein and nonprotein amino acids serve as substrates for a biochemical pathway of at least five steps: amino acid (1)⟶ N-hydroxyamino acid (2)⟶ aldoxime (3)⟶ thiohydroximate (4)⟶ desulfoglucosinolate (5)⟶ glucosinolate. Enzymatic activities capable of catalyzing steps (1), (2), (4), and (5) have been reported (8, 12, 14, 20, 21).

In contrast to our knowledge of glucosinolate chemistry and biochemistry, very little is known about the genes which encode the biosynthetic enzymes (6). To this end, we have initiated a program in biochemical genetics of glucosinolate metabolism in *Arabidopsis thaliana* with the purposes of better defining the glucosinolate biosynthetic pathway, establishing the relationship between leaf and seed glucosinolate biosynthesis, determining which genes, if any, are common to the biosynthesis of all glucosinolates, and evaluating the physiological effects of blocking specific biosynthetic steps. Such information is a prerequisite to our long-term goal of using molecular genetics to manipulate glucosinolate levels in crop species.

We have recently characterized seed and leaf glucosinolates of *A. thaliana* (11). The chemistry of the 23 different glucosinolates that have been identified (Table I) and the biochemical analyses done in other glucosinolate bearing species, make it possible to predict that all are derived from just three protein amino acids. Three glucosinolates (Table I; glucosinolates 16, 17, 18) contain a 3-indolylmethyl nucleus that could originate from tryptophan (13, 16, 19). The 2-phenylethyl glucosinolate

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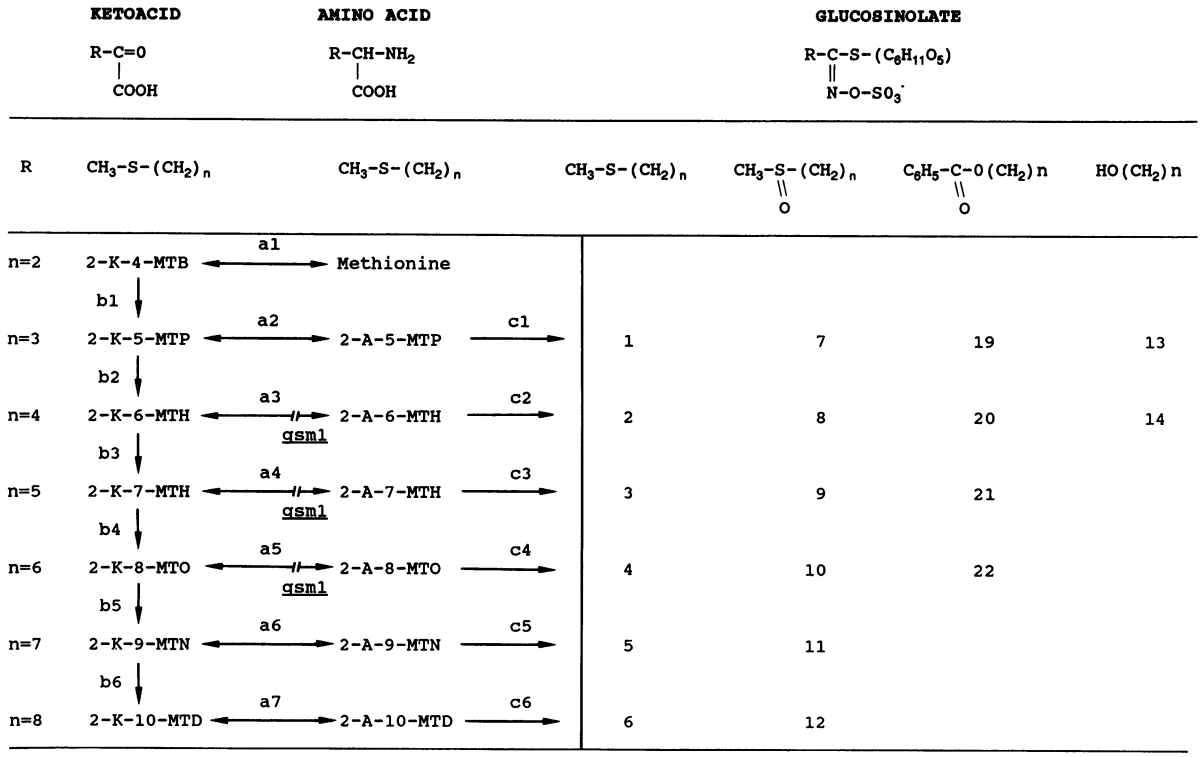


Figure 1. Hypothetical pathway for the biosynthesis of glucosinolates from methionine in tissues of *A. thaliana*. Compounds shown in the biosynthetic pathway for the aliphatic glucosinolates (keto acids, amino acids, and glucosinolates) vary with respect to side groups on (R, horizontal axis) and the overall length (*n*, vertical axis) of their carbon side-chain. The arrows denote one or more enzymatically catalyzed reactions: a, transamination; b, carbon-chain elongation pathway; c, conversion of an amino acid to a methylthioglucosinolate and subsequent modification of the carbon side-chain. The direction of the arrow indicates the normal direction of carbon flow. The glucosinolate numbers refer to those given in Table I. 2-A-8-MTO, 2-amino-8-methylthiooctanoic acid; 2-A-9-MTN; 2-amino-9-methylthiononanoic acid; 2-A-10-MTD, 2-amino-10-methylthiodecanoic acid; 2-K-4-MTB, 2-keto-4-methylthiobutanoic acid; 2-K-5-MTP, 2-keto-5-methylthiopentanoic acid; 2-K-6-MTH, 2-keto-6-methylthiohexanoic acid; 2-K-7-MTH, 2-keto-7-methylthioheptanoic acid; 2-K-8-MTO, 2-keto-8-methylthiooctanoic acid; 2-K-9-MTN, 2-keto-9-methylthiononanoic acid; 2-K-10-MTD, 2-keto-10-methylthiodecanoic acid. The putative position of the *gsm1* metabolic block (a3, a4, a5) is indicated by //.

found in only trace amounts in *A. thaliana* may be derived from phenylalanine (25; Table I, 23). Methionine is probably the precursor for the remaining 19 glucosinolates (see Fig. 1). The ω-methylthio glucosinolates (Table I; glucosinolates 1–6) may be derived from methionine by way of a chain-extension pathway (26) to produce a homologous series of amino acids ($R = CH_3S(CH_2)_n$; $n = 3$–8) which, in turn, serve as precursors for glucosinolate biosynthesis. Additional modification of aliphatic side chains could generate the corresponding series of ω-methylsulfinyl glucosinolates (Table I, 7–12), the benzoyloxy glucosinolates (Table I, 19–22), 3-hydroxypropyl and 4-hydroxybutyl (Table I, 13, 14) and 3-butenyl glucosinolates (Table I, 15).

In this paper we describe the isolation of mutants with altered glucosinolate content by HPLC screening and the genetic and biochemical characterization of one such mutant. The significance of this work with respect to the elucidation of the glucosinolate biosynthetic pathway is discussed.

MATERIALS AND METHODS

Lines and Growth Conditions

All lines of *A. thaliana* used in this study were derived from the Columbia ecotype. Line MSU8 (*gl1*) has been described previously (9). Plants were grown in growth chambers under the following conditions: soil = Terra-lite Redi-earth prepared by WR Grace and Co. Canada Ltd. (Ajax, Ontario, L1S 3C6); subirrigation, 16 or 18 h photoperiod, 100–150 $\mu E/m^2/s$ (PAR) of cool-white fluorescent light supplemented with incandescent light, and 22°C.

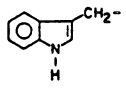
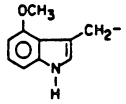
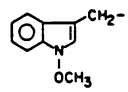
Mutagenesis (10), cross-fertilization, and handling of *A. thaliana* seed (22) has been described.

Analysis of Glucosinolate Content in *A. thaliana*

The extraction of glucosinolates from bulk leaf (rosettes from 10 plants/sample) and seed (2 mg samples) tissue, their conversion to desulphoglucosinolates, subsequent separation by HPLC, and verification by thermospray LC/MS[3] was done as described by Hogge *et al.* (11). Subsequent identification of desulphoglucosinolates was based on HPLC elution time.

[3] Abbreviations: LC-MS, liquid chromatography-mass spectrometry; 2-A-5-MTP, 2-amino-5-methylthiopentanoic acid; 2-A-6-MTH, 2-amino-6-methylthiohexanoic acid; 2-A-7-MTH, 2-amino-7-methylthioheptanoic acid; 2-A-8-MTO, 2-amino-6-methylthiooctanoic acid; M2, progeny of the mutagenized generation; M3, two generations following mutagenesis.

Table I. *Glucosinolates[a] Identified in A. thaliana Leaf and Seed Tissues*

Aliphatic Glucosinolates	Structure of 'R' Group	Aromatic Glucosinolates	Structure of 'R' Group
With methylthioalkyl side-chains		With Heterocyclic side-chains	
1. 3-Methylthiopropyl	CH_3-S-$(CH_2)_3$-	16. 3-Indolylmethyl	
2. 4-Methylthiobutyl	CH_3-S-$(CH_2)_4$-		
3. 5-Methylthiopentyl	CH_3-S-$(CH_2)_5$-		
4. 6-Methylthiohexyl	CH_3-S-$(CH_2)_6$-		
5. 7-Methylthioheptyl	CH_3-S-$(CH_2)_7$-	17. 4-Methoxy-3-indolylmethyl	
6. 8-Methylthiooctyl	CH_3-S-$(CH_2)_8$-		
With methylsulphinylalkyl side-chains			
7. 3-Methylsulphinylpropyl	CH_3-SO-$(CH_2)_3$-	18. 1-Methoxy-3-indolylmethyl	
8. 4-Methylsulphinylbutyl	CH_3-SO-$(CH_2)_4$-		
9. 5-Methylsulphinylpentyl	CH_3-SO-$(CH_2)_5$-		
10. 6-Methylsulphinylhexyl	CH_3-SO-$(CH_2)_6$-		
11. 7-Methylsulphinylheptyl	CH_3-SO-$(CH_2)_7$-	With nonhetrocyclic side-chains	
12. 8-Methylsulphinyloctyl	CH_3-SO-$(CH_2)_8$-	19. 3-Benzoyloxypropyl	C_6H_5COO-$(CH_2)_3$-
		20. 4-Benzoyloxybutyl	C_6H_5COO-$(CH_2)_4$-
		21. 5-Benzoyloxypentyl	C_6H_5COO-$(CH_2)_5$-
With other side-chains		22. 6-Benzoyloxyhexyl	C_6H_5COO-$(CH_2)_6$-
13. 3-Hydroxypropyl	HO-(CH_2)3-	23. 2-Phenylethyl	C_6H_5-$(CH_2)_2$-
14. 4-Hydroxybutyl	HO-(CH_2)4-		
15. 3-Butenyl	CH_2=CH-$(CH_2)_2$-		

[a] Numbers assigned to glucosinolates are used in tables, figures, and text.

The glucosinolate content of different tissues was quantitated on the basis of UV absorbance relative to that of a standard (*o*-nitrophenyl-β-D-galactopyranoside) after adjusting for differences in UV absorption response (response factor). Response factors were determined by the thymol method (1) for representative compounds.

For analysis of leaf glucosinolate content of individual plants, the four oldest leaves were removed when the plants were 2 to 3 weeks old (immediately prior to bolting; only samples of 6 mg or more were used), weighed, and immediately boiled in 0.75 mL of hot water for 10 min and the supernatant was added directly to the DEAE-Sephadex A-25 (pyridine acetate form) column and analyzed by HPLC.

Precursor Feedings

Labeled compounds [2-^{14}C]acetate (170 μCi/μmol), and [2-^{14}C]methionine (4.139 μCi/μmol) obtained from commercial sources (New England Nuclear), and [2-^{14}C]2-A-5-MTP (0.290 μCi/μmol), [2-^{14}C]2-A-6-MTH (0.198 μCi/μmol), and [2-^{14}C]2-A-7-MTH (0.228 μCi/μmol) prepared previously (3, 4), were administered to 3-week-old plants (prior to bolting) as follows. Roots were excised under water with a scalpel and the cut end of the shoots immersed in a 50 μL aqueous solution of one of the compounds (1 μmol) in the tip of a 1.5 mL microfuge (feeding) tube. Following uptake of the solution (approximately 1 h), three 50 μL aliquots of water were added and allowed to be absorbed in succession (each wash was approximately 1 h). Uptake of the compounds was calculated by determining the remaining radioactivity in the tube. The shoots were then transferred to a 1.5 mL microfuge tube containing 1 mL of water enclosed inside a tray with transparent lid (to prevent desiccation) and incubated for 20 to 22 h in a growth chamber (22°C, 100 μE/m^2/s continuous light). Each compound was fed separately to six plants. The six plants were bulked, the glucosinolates extracted, converted to desulphoglucosinolates, and separated by HPLC. The effluent from the column was analyzed by UV spectroscopy and assayed for radioactivity by mixing with scintillant (Flo-Scint III, Radiomatic Instruments and Chemical Co., Tampa, FL) at a rate of 1.5 mL/min to give a total flow rate of 2 mL/min upon entering the Flo-One detector.

RESULTS

Mutant Isolation

Leaf tissue of *A. thaliana* contains a characteristic array of glucosinolates which can be detected by converting to their desulfo analogs and separating by liquid chromatography (11). We sought to isolate mutants defective in glucosinolate metabolism by screening, individually, large numbers of ethyl methane sulfonate mutagenized plants (M2 generation) for those with a glucosinolate profile that differed significantly from that of the wild type. To facilitate the screening, we modified the extraction procedure previously reported (11) such that the glucosinolate content of leaves of individual plants could be more rapidly determined without sacrificing the plants. The oldest four leaves of a plant were harvested (sample sizes were between 6 and 500 mg) and the glucosinolates extracted in boiling water without tissue homogenization. The HPLC chromatograms of glucosinolates extracted in this way (Fig. 2A) were similar to those of glucosinolates extracted from bulk tissue (*cf.* Fig. 2A with Fig. 3A). Although the amount of total glucosinolates varied greatly from plant to plant (standard deviation was approximately 50% of the mean, data not shown), the amounts of individual glucosinolates relative to each other was constant. Thus, mutants

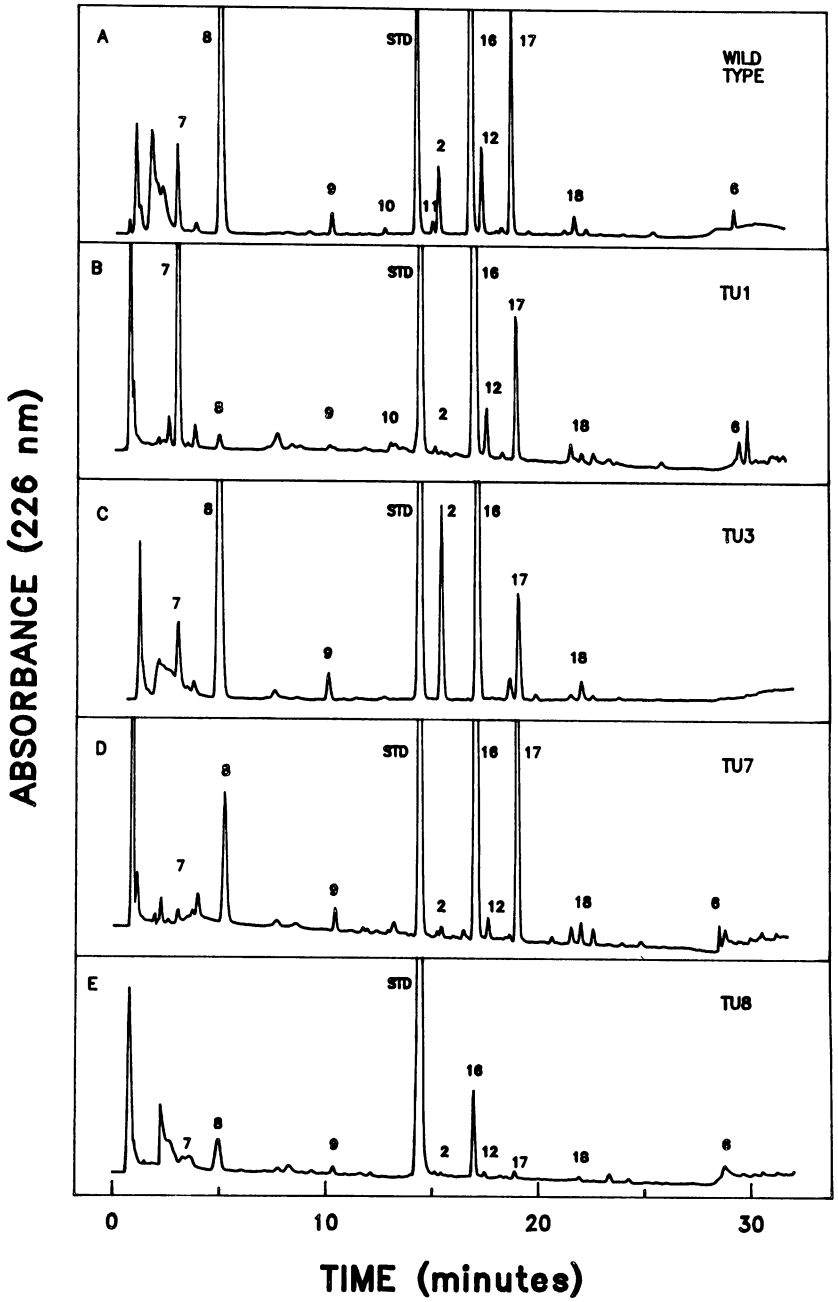


Figure 2. HPLC chromatograms of desulfoglucosinolates from leaf tissue of individual *A. thaliana* plants from wild-type (A) and mutant (B–E) lines. Glucosinolates were extracted from the tissue, separated by HPLC, and detected by absorption at 226 nm. Specific desulfoglucosinolates are identified by numbers which correspond to those used in Table I. Identification was based on elution time relative to the standard (STD) *o*-nitrophenyl-β-D-galactopyranoside.

with decreased amounts of one or more of the leaf glucosinolates could be easily distinguished from wild type.

The glucosinolate content of leaves of approximately 1200 M2 plants from an ethyl methane sulfonate mutagenized M2 population was determined. The desulphoglucosinolate HPLC chromatograms of 24 plants appeared to be significantly different from wild type. The 24 putative mutants were allowed to self-fertilize and the glucosinolate contents of 5 to 10 progeny from each were determined. For 6 of the 24 putative mutants, all M3 progeny tested had an altered leaf glucosinolate content similar to their immediate parent. These data suggest that the traits are genetically inherited (see also "Genetic Analysis of Line TU1" below and "Discussion") and that the mutants isolated were homozygous for the mutation(s). The HPLC chromatograms of four of these mutants are given in Figure 2. A fifth (TU5) and a sixth (TU6) line were phenotypically similar to line TU1 (Fig. 2B) and line TU3 (Fig. 2C), respectively.

Glucosinolate Content of Line TU1

Line TU1 appeared to have greatly reduced amounts of several glucosinolates with aliphatic side chains including the most abundant in leaf tissue, 4-methylsulphinylbutyl glucosinolate (Fig. 3B, glucosinolate 8). We chose this line for characterization because the initial isolate was healthy and the phenotype did not vary greatly from plant to plant. Glucosinolates in bulked leaf and seed tissue were determined

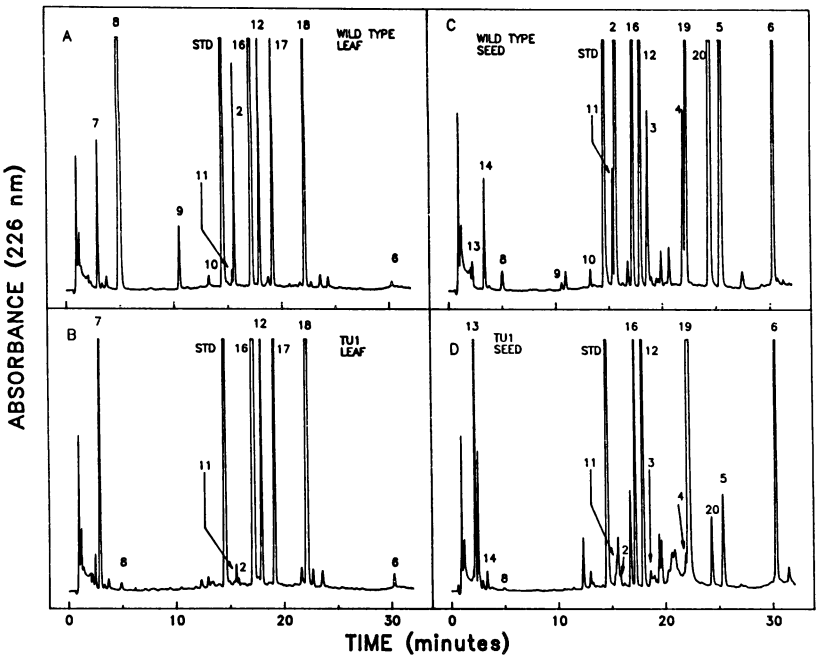


Figure 3. HPLC chromatograms of desulfoglucosinolates from bulked leaf (A, B) and seed tissue (C, D) of wild type (A, C) and plants of mutant line TU1 (B, D). Glucosinolates were extracted from the tissue, separated by HPLC, and detected by absorption at 226 nm. Specific desulfoglucosinolates are identified by numbers which correspond to those used in Table I. Identification was based on elution time relative to the standard (STD) *o*-nitrophenyl-β-D-galactopyranoside and analysis by mass spectrometry.

by HPLC as described in "Materials and Methods" (Fig. 3; Table II). The identities of the desulphoglucosinolates were verified using LC-MS (data not shown). Of the 19 glucosinolates that could be detected by our HPLC analysis, line TU1 had greatly reduced amounts of the eight aliphatic glucosinolates that had a side-chain carbon length (*n*) of 4, 5, and 6 (glucosinolates 2, 3, 4, 8, 9, 10, 14, and 20; Table II). In addition, the mutant contained higher than wild-type levels of aliphatic glucosinolates where $n = 3$ (7, 13, and 19; see Table II). These results suggest that TU1 has a metabolic block in the biosynthetic pathway for aliphatic glucosinolates (Fig. 1).

Genetic Analysis of Line TU1

To determine the mode of inheritance of the TU1 glucosinolate phenotype, reciprocal crosses were made between line TU1 and line MSU8 (*gl1*). The F_1 and F_2 progeny were scored for TU1 or wild-type glucosinolate content (gsm and GSM, respectively) in leaf tissue and, as a control the recessive nuclear marker *gl1* (lack of leaf trichomes). The F_1 progeny from both crosses had a wild-type phenotype while the F_2 progeny were approximately 9 GSM, *GL1/_*: 3 GSM, *gl1/gl1*: 3 gsm, *GL1/_*: 1 gsm, *gl1/gl1* (Table III). These data suggest that the TU1 leaf glucosinolate phenotype is due to a recessive allele of a single nuclear gene (designated *gsm1-1* for *g*lucosinolate *m*etabolism) which segregates independently of the *GL1* gene.

Three lines of evidence support the hypothesis that the novel seed glucosinolate content of line TU1 is also due to the *gsm1-1* allele and not to an independent mutation. First, *gsm1-1\gsm1-1* F_2 plants from each of the crosses TU1 × MSU8 and MSU8 × TU1 produced seed with a glucosinolate content identical to that of line TU1. Second, the TU1 seed and leaf glucosinolate phenotypes cosegregated through six backcrosses to wild type. Third, line TU5 is homozygous for an independently derived recessive allele of the *GSM1* gene. The glucosinolate content of both leaf and seed tissues of line TU5 are similar to line TU1 (data not shown).

The glucosinolate content of F1 seed (batches of 100) from the crosses of lines TU1 and MSU8 were also examined (Table III). As expected, F1 seed from the cross MSU8 × TU1 had a wild-type seed glucosinolate content. In contrast, F1 seed from the reciprocal cross (TU1 × MSU8) had a TU1-like glucosinolate content suggesting that the seed glucosinolate phenotype is under maternal control.

In Vivo Labeling of Glucosinolates With ^{14}C Intermediates

The nature of the phenotype of line TU1 suggests that the *GSM1* gene encodes an enzyme common to the biosynthesis of several of the aliphatic glucosinolates. We attempted to determine more about the biosynthetic pathway and the position of this metabolic block by feeding five different 2-C^{14}-labeled, putative-precursors of aliphatic glucosinolates to wild type and TU1 (*gsm1-1/gsm1-1*) shoots: acetate and amino acids, methionine (Fig. 1, $n = 2$), 2-A-5-MTP (Fig. 1, $n = 3$), 2-A-6-MTH (Fig. 1, $n = 4$), and 2-A-7-MTH (Fig. 1, $n = 5$). The glucosinolates from the fed shoots were separated by HPLC and analyzed by UV absorption and flow through scintillation counter. Uptake of the fed compounds by the shoots was between 45.4 and 79.1%, while the recovery in the glucosinolate fraction was: acetate: 1.5% (wild type), 0.5% (TU1); methionine: 3.2% (wild type), 1.6% (TU1); 2-A-5-MTP: 4.6% (wild type), 9.0% (TU1); 2-A-6-MTH: 4.2% (wild type), 6.5% (TU1); 2-A-7-MTH: 5.0% (wild type), 11.0% (TU1). As expected, only the aliphatic glucosinolates were radiolabeled. Radioactivity was never found in the indole glucosinolate fractions.

The radioactivity incorporated into individual aliphatic glucosinolates in shoots of wild type and line TU1 are shown in Figures 4 and 5, respectively. These results are consistent

Table II. *Glucosinolate Content of A. thaliana Leaf and Seed Tissue of Wild-Type and Mutant Lines*

Individual measurements of leaf glucosinolate content were made on the bulked tissue from five shoots (0.290 g). Eight such measurements were made for both the mutant (TU1) and wild type (W.T.). For the determination of seed glucosinolates, seven seed samples of approximately 1 mg each were used.

No.	Glucosinolate	Response Factor[a]	Leaf				Seed			
			W.T.	S.D.	TU1	S.D.	W.T.	S.D.	TU1	S.D.
			nmol/mg		*nmol/mg*		*nmol/mg*		*nmol/mg*	
2.	4-Methylthiobutyl	1.0	0.277	0.035	0.006	0.002	4.370	1.470	0.347	0.143
3.	5-Methylthiopentyl	1.0	ND		ND		1.260	0.237	0.269	0.060
4.	6-Methylthiohexyl	1.0	NS		ND		NS		ND	
5.	7-Methylthioheptyl	1.0	ND		ND		5.310	1.510	2.620	0.217
6.	8-Methylthiooctyl	1.0	0.043	0.013	0.044	0.010	6.900	1.330	11.500	1.090
7.	3-Methylsulphinylpropyl	1.0	0.152	0.075	0.476	0.149	0.350	0.218	0.309	0.162
8.	4-Methylsulphinylbutyl	1.0	1.200	0.592	0.018	0.005	1.010	0.828	ND	
9.	5-Methylsulphinylpentyl	1.0	0.056	0.019	ND		0.159	0.098	ND	
10.	6-Methylsulphinylhexyl	1.0	0.020	0.002	ND		0.220	0.116	ND	
11.	7-Methylsulphinylheptyl	1.0	0.043	0.009	0.025	0.005	1.270	0.515	0.801	0.354
12.	8-Methylsulphinyloctyl	1.0	0.300	0.042	0.290	0.035	6.930	1.770	10.800	1.070
13.	3-Hydroxypropyl	1.48	ND		ND		0.368	0.104	5.080	1.180
14.	4-Hydroxybutyl	1.48	ND		ND		3.120	0.848	0.439	0.080
16.	3-Indolylmethyl	0.25	0.341	0.045	0.462	0.100	1.270	0.211	2.820	0.354
17.	4-Methoxy-3-indolylmethyl	0.25	0.126	0.036	0.089	0.019	ND		ND	
18.	1-Methoxy-3-indolymethyl	0.25	0.089	0.021	0.140	0.038	ND		ND	
19.	3-Benzoyloxypropyl	0.41	ND		ND		2.020	0.364	21.500	1.280
20.	4-Benzoyloxybutyl	0.41	ND		ND		7.310	1.320	0.744	0.058

[a] Response factors were determined by the thymol method for glucosinolates 2, 12, 14, 16, and 20 only. Glucosinolates with structures similar to those tested were assumed to have similar response factors as suggested by Buchner (2). ND, Not detectable; NS, detectable but peak was not well enough resolved for quantitative measurement.

with the proposed pathway for glucosinolate biosynthesis in Brassicaceae (26; Fig. 1) as follows. First, when methionine, acetate, and 2-A-5-MTP were fed, all detectable aliphatic glucosinolates produced by the shoots were radiolabeled (glucosinolates 2, 7, 8, 9, 10, 11, and 12 in wild type, Fig. 4, A–C; data not shown for line TU1) suggesting that all three compounds are precursors of all of the aliphatic glucosinolates. Second, the proportion of radioactivity incorporated into glucosinolates of carbon side-chain length *n* was highest when shoots were fed an amino acid with a carbon side-chain length *n*. For example, when wild-type shoots were fed 2-A-7-MTH ($n = 5$), a higher proportion of the radioactivity recovered in the glucosinolate fraction was associated with glucosinolates 3 and 9 ($n = 5$; Fig. 4E) than when any other compound was fed. Indeed, glucosinolate 3 which is not normally detectable in leaf tissue (Table II) was synthesized when shoots were fed 2-A-7-MTH (Fig. 4E, Fig. 5C). These data suggest that amino acids of carbon length *n* are limiting substrates for biosynthesis of all glucosinolates of carbon length *n*. Third, shoots fed amino acids of carbon chain length *n* were found to have radiolabeled aliphatic glucosinolates of carbon chain length greater than, but not less than, *n*. For example, wild-type shoots fed 2-A-6-MTH ($n = 5$) contained radiolabeled glucosinolates 9 ($n = 5$) and 12 ($n = 8$) but not 7 ($n = 3$) (Fig. 4D), despite the fact that glucosinolate 7 was the second most abundant glucosinolate in the shoots (data not shown). Similarly, when TU1 shoots were fed 2-A-7-MTH no label was observed in 3-methylsulphinylpropyl glucosinolate (Fig. 5C) although it was the second most abundant aliphatic glucosinolate produced in the leaves. These data are

Table III. *Genetic Characterization of Mutant TU1*

Each plant was scored for the presence (GL1) or absence (gl1) of leaf hairs and the glucosinolate content as measured by reverse-phase HPLC; either wild type (*GSM*) or TU1 (*gsm*) like.

Cross		Phenotype[a]				X^2
		GSM,GL1	*gsm*,GL1	*GSM*,gl1	*gsm*,gl1	
		Obs\Exp[b]	Obs\Exp[b]	Obs\Exp[b]	Obs\Exp[b]	
MSU8 × TU1						
	F1	7	0	0	0	
	F2	44\43.2	10\14.4	16\14.4	6\4.8	1.83
TU1 × MSU8						
	F1	2	0	0	0	
	F2	57\48.6	11\16.2	20\16.2	5\5.4	4.04

[a] Data expressed as number of plants. [b] Obs, observed numbers of plants of the indicated phenotype; Exp, expected numbers of plants of the indicated phenotype assuming segregation of two unlinked loci and complete dominance.

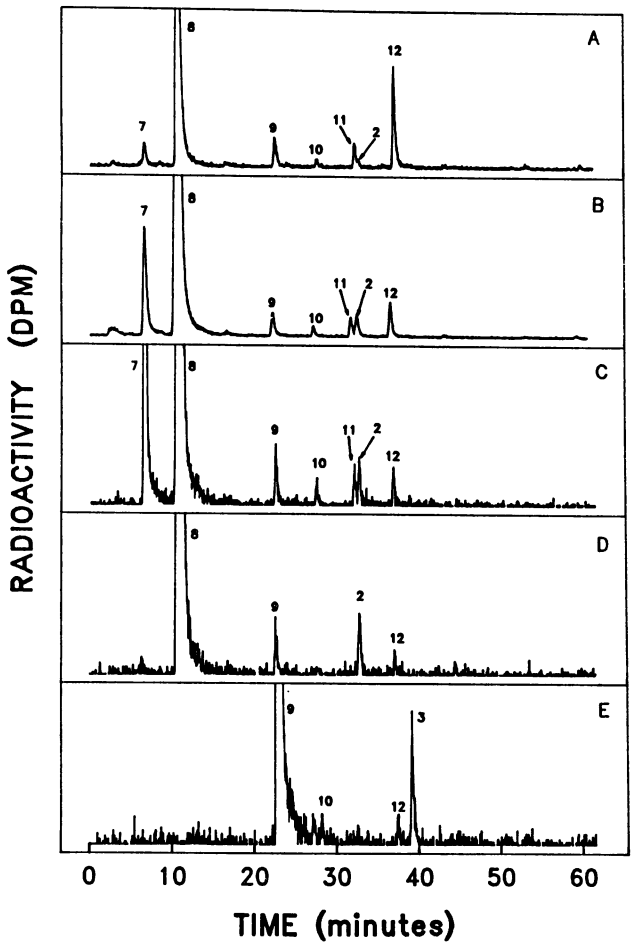


Figure 4. Radioactivity incorporated into glucosinolates after feeding wild-type shoots with radiolabeled compounds. For each of five different compounds fed, six plants were allowed to take up and metabolize the 1 μmol of the indicated compound during a 24 h period. The plants were harvested in bulk, and the glucosinolates were extracted from the tissue, separated by HPLC, and detected by absorption at 226 nm. Radioactivity in each fraction was detected by liquid scintillation. Specific desulfoglucosinolates are identified by numbers which correspond to those used in Table I. Identification was based on elution time relative to the standard (STD) *o*-nitrophenyl-β-D-galactopyranoside. The vertical scale for each graph has been normalized with respect to the total number of counts taken up by shoots in different feedings (adjusts for variation in uptake and specific activity of the five compounds). A, Shoots fed acetate; B, shoots fed methionine; C, shoots fed 2-A-5-MTP; D, shoots fed 2-A-6-MTH; E, shoots fed 2-A-7-MTH.

expected if, as proposed (Fig. 1), the aliphatic glucosinolates are biosynthesized from methionine by successive additions of acetate-derived carbon atoms.

Shoots of line TU1 synthesize less than wild-type levels of glucosinolates 2, 8, and 9 (Fig. 3B). Figure 6 shows the HPLC chromatograms of glucosinolates (absorption at 226 nm) from shoots of TU1 plants fed 1 μmol of the five compounds indicated. Shoots that were fed three of the compounds (acetate, methionine, 2-A-5-MTP; Fig. 6, A–C, respectively) were not different in glucosinolate content from shoots fed only water (data not shown; *cf.* Fig. 6, A–C to Fig. 3B). By contrast, the feeding of 2-A-6-MTH and 2-A-7-MTH resulted in an increase of the synthesis of glucosinolates 2, 8, and 9, respectively (*e.g.* in Fig. 6, *cf.* the level of incorporation in the glucosinolate 8 fraction between plants fed 2-A-5-MTP, Fig. 6C *versus* 2-A-6-MTH, Fig. 6D) suggesting that in the mutant these amino acids are limiting substrates and lie downstream of the TU1 metabolic block. Using the same reasoning one can conclude that methionine and 2-A-5-MTP are not limiting and, therefore, lie upstream of the metabolic block. The observed increase in the steady-state levels of $n = 3$ glucosinolates in the TU1 mutant is consistent with this conclusion.

Growth Analysis

Blocks in biosynthetic pathways can result in detrimental effects on plant growth because of the build-up of toxic intermediates or the loss of end products. The effect of the *gsm1-1* allele on shoot growth was tested as follows. Flats were planted with alternating rows of wild-type and TU1 plants and kept at 4°C for 7 d to ensure uniformity in germination. Afterward, plants were shifted to normal growth conditions (d 1). On and between d 10 and 25, five plants per line were harvested every 3 d and the fresh weight determined for individual plants. Under these conditions, no significant difference in growth between line TU1 and wild type was detected.

DISCUSSION

Twenty-three different glucosinolates have been identified in leaf and seed tissue of *A. thaliana* (11). The biosynthesis of 19 of these, the aliphatic and benzoyloxy-glucosinolates, can be explained by postulating a single pathway with methionine as a precursor, as illustrated in Figure 1 (see also ref. 26).

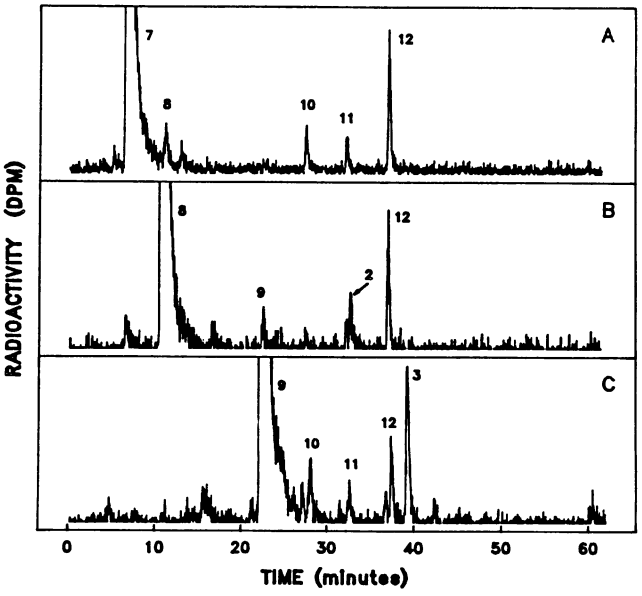


Figure 5. Radioactivity incorporated into glucosinolates after feeding TU1 shoots with radiolabeled compounds. The analysis was done as described in Figure 4. A, Shoots fed 2-A-5-MTP; B, shoots fed 2-A-6-MTH; C, shoots fed 2-A-7-MTH.

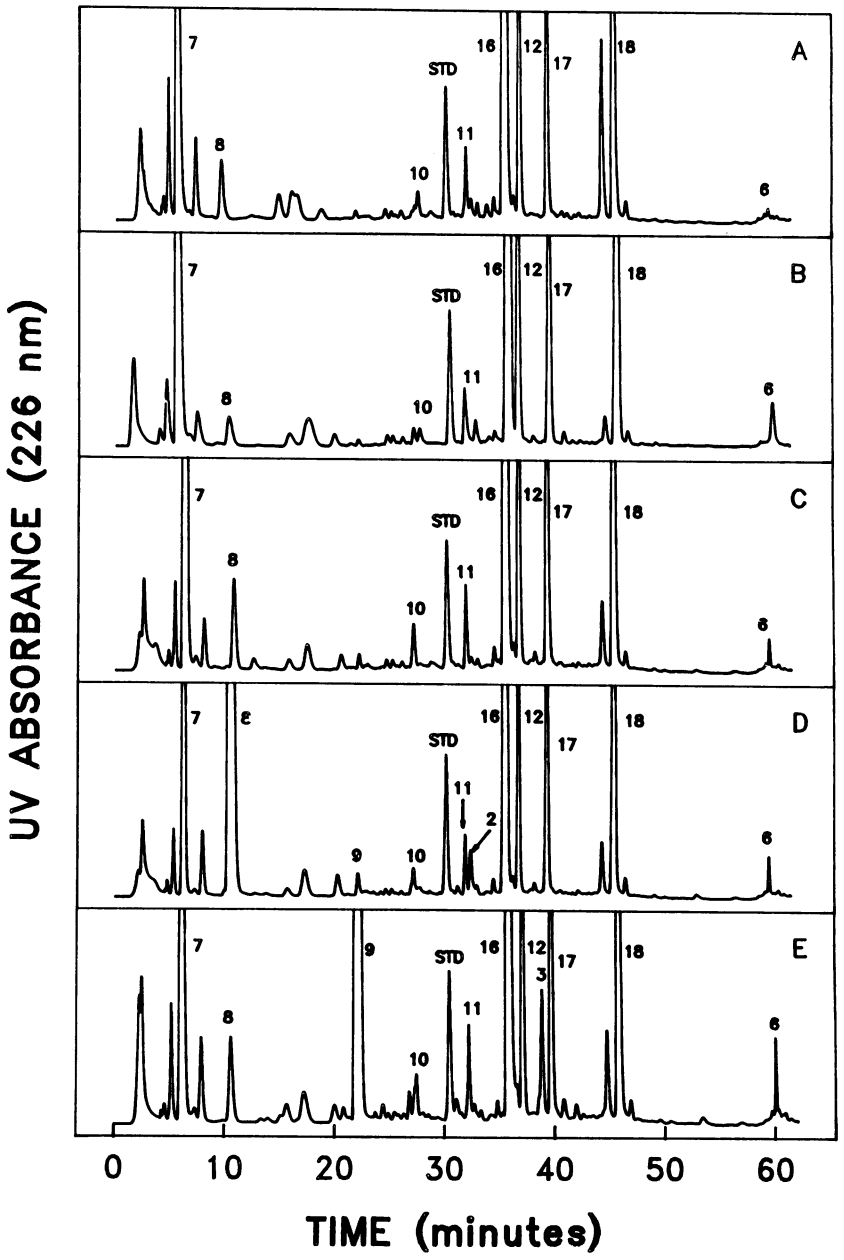


Figure 6. Glucosinolates in TU1 shoots after feeding various compounds. The analysis was done as described in Figure 4. Specific desulfoglucosinolates are identified by numbers which correspond to those used in Table I. Identification was based on elution time relative to the standard (STD) *o*-nitrophenyl-β-D-galactopyranoside. Note that graphs C, D, and E correspond to the same feedings as in Figure 5, A, B, and C respectively. A, Shoots fed acetate; B, shoots fed methionine; C, shoots fed 2-A-5-MTP; D, shoots fed 2-A-6-MTH; E, shoots fed 2-A-7-MTH.

Deamination of methionine (Fig. 1, step *a1*) and multiple rounds of carbon chain-extension using acetate as a donor (Fig. 1, step *b1–b6*) produces a homologous series of ketoacids which vary only in the length of the carbon side chain. The ketoacids are then transaminated and the glucosinolate moiety synthesized to produce a homologous series of methylthio glucosinolates (Fig. 1, steps *c1–c6*). The methylthio glucosinolates, in turn, can be substrates for several enzymes which modify the methylthio side chain to produce the -methyl sulfinyl, -hydroxy, and benzoyloxy glucosinolates (Fig. 1, steps *c1–c6*).

Several lines of evidence presented here are consistent with the hypothesis that glucosinolate biosynthesis in *A. thaliana* occurs by pathways similar to those described above. First, methionine and acetate are precursors of aliphatic but not indole glucosinolates. Second, an increase or decrease in biosynthesis of an aliphatic glucosinolate either through feeding or the effects of the *gsm1–1* mutation is paralleled by a similar change in other glucosinolates of the same carbon side-chain length. Third, amino acids 2-A-6-MTH ($n = 4$) and 2-A-7-MTH ($n = 5$) are precursors for glucosinolates with a carbon side-chain length equal to or greater but not less than their own.

We have isolated six *A. thaliana* mutants with an altered glucosinolate composition. Two factors facilitated the isolation of the mutants, despite the laborious nature of the screen (approximately 100 plants assayed/week). First, the relative amounts of aliphatic glucosinolates were found to vary little from plant to plant, thus making mutants in glucosinolate metabolism more readily recognizable. Second, the high fre-

quency of mutations obtainable in ethyl methane sulfonate mutagenized populations of *A. thaliana* (10) eliminates the need to screen more than a few thousand plants to have a high probability of isolating a given mutant. It should be possible to use an HPLC screen to isolate mutants of *A. thaliana* with lesions in the biochemical pathways of many nonessential secondary metabolites.

We have analyzed one of these mutant lines (TU1) in detail. Line TU1 is unable to synthesize wild-type levels of many aliphatic glucosinolates because it is homozygous for a recessive mutant allele (*gsm1-1*) of the nuclear gene *GSM1*. It is likely, therefore, that the product of *GSM1* is required for an enzymatic step in the glucosinolate biosynthetic pathway and that the *gsm1-1* allele results in partial loss of function. Although experiments reported here have not identified the exact metabolic block in TU1, only a few possibilities exist if we assume that, in general, the biosynthetic pathway proposed in Figure 1 is correct. We can deduce, from the feeding experiments, that the *GSM1* product is not required for any biosynthetic step prior to 2-A-5-MTP nor for any step between amino acids 2-A-5-MTP, 2-A-6-MTH, and 2-A-7-MTH and their corresponding glucosinolates $n = 3$, 4, and 5 (Fig. 1, steps *c1–c3*). The biosynthesis of glucosinolates 11 and 12 ($n = 7$, 8, respectively) appears to be relatively unaffected in line TU1. Because the biosynthesis of glucosinolates 11 and 12 depend on the activity of all of the enzymes involved in chain extension (Fig. 1, steps *b1–b6*) the *GSM1* gene product could not be required for any chain-extension reactions. The TU1 metabolic block must, therefore, be involved in the biosynthesis of 2-A-6-MTH, 2-A-7-MTH, and 2-A-8-MTO from their respective ketoacids (steps *a3–a5*, Fig. 1).

The GSM1 gene product could be: an intracellular transporter of ketoacids as would be needed if carbon-chain extension and transamination occur in different cellular compartments; a transaminase which recognizes as a substrate ω-methylthio ketoacids of only specific carbon-chain lengths. The hypothesis could be tested by synthesizing and feeding the appropriate ketoacids to TU1 and wild-type lines. It should be noted that radioactivity was detected in the glucosinolate 12 fraction after feeding TU1 plants 2-A-6-MTH and 2-A-7-MTH indicating that TU1 plants have the capability of deaminating those amino acids; or a protein needed for release of the ketoacid from a carbon-chain extension enzyme complex. Such a hypothesis assumes that all the observed carbon-chain extension is catalyzed by a single enzyme complex which uses the ketoacid it synthesizes as a substrate for a subsequent series of reactions (analogous to channeling in the biosynthesis of cyanogenic glucosides, 21; or the fatty-acid synthetase complex, 23).

Levels of both $n = 3$ and $n = 8$ glucosinolates in shoots of TU1 are not decreased in plants homozygous for the *gsm1-1* allele. These data suggest that the transamination occurring in steps *a2*, *a6*, and *a7* must be catalyzed by the products of genes other than *GSM1*. Lines TU3 and TU6, which have lower than wild-type levels of $n = 8$ glucosinolates 6 and 12, may be deficient for a transaminase required for step *a7* (Fig. 1).

The *gsm1-1* allele affects the glucosinolate content of both leaf and seed tissues similarly. However, the seed phenotype, unlike that of the leaf, is inherited as a maternal effect. Control of seed glucosinolates by the maternal genotype has been reported previously (*e.g. Brassica napus*, 15, 18). Such data indicate that biosynthesis of leaf glucosinolates is dependent on enzymes encoded by the zygotic genome, whereas the biosynthesis of seed glucosinolate is dependent on enzymes encoded by the maternal genome. The biosynthesis of seed glucosinolates could involve the transport of glucosinolate biosynthetic enzymes, the RNA encoding those enzymes, or glucosinolates themselves, from the maternal tissues to the embryo. Consistent with the latter hypothesis are recent physiological studies that have suggested that embryos of *B. napus* developing *in vitro* acquire their glucosinolates from the exogenous medium rather than synthesizing them *de novo* (5, 7).

If seed glucosinolates are indeed derived from maternal tissue, two important predictions can be made which relate to the manipulation of glucosinolate content in plants. First, any genetic alterations which eliminate activity of an enzyme in the glucosinolate biosynthetic pathway and, therefore, decrease leaf glucosinolate content, should also decrease the seed glucosinolate content. Second, it may be possible to eliminate seed glucosinolates specifically, by genetically altering any protein required for glucosinolate transport to the embryo.

The five mutants isolated in addition to line TU1 have not been characterized extensively. Preliminary results indicate that the glucosinolate phenotypes of lines TU3, TU5, and TU7 all segregate as single recessive Mendelian genetic markers. Line TU5, with a similar leaf and seed glucosinolate content to line TU1 failed to complement the *gsm1-1* allele and thus represents a new allele, *gsm1-2*. Line TU8 has low levels of all leaf glucosinolates but normal levels of seed glucosinolates and morphologically is a dwarf with underdeveloped leaves. The glucosinolate and dwarf phenotypes cosegregate. Leaf tissue of line TU7 contains low levels of all aliphatic glucosinolates, the absolute amounts of which are variable between individual plants. Metabolite feeding experiments, similar to the ones described here for TU1, have failed to identify any differences between line TU7 and wild type.

At present, our screen for mutants has identified only a small number of the genes that one might predict would be involved in glucosinolate biosynthesis. This is not unusual considering the small number of plants screened and estimates of mutation frequencies in our mutagenized stocks (10). A recent screen of M3 seed lines has identified several additional putative mutants including one that does not make ω-benzoyloxyglucosinolates. Additional screening, therefore, should identify additional mutants with altered glucosinolate biosynthesis.

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