

## Characterization of the *Arabidopsis* TU8 glucosinolate mutation, an allele of *TERMINAL FLOWER2*

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Received 16 February 2004; accepted in revised form 31 March 2004

**Key words:** *Arabidopsis*, chromodomain protein, glucosinolate, regulation, TFL2, TU8

### Abstract

Glucosinolates are a group of defense-related secondary metabolites found in *Arabidopsis* and other cruciferous plants. Levels of leaf glucosinolates are regulated during plant development and increase in response to mechanical damage or insect feeding. The *Arabidopsis* TU8 mutant has a developmentally altered leaf glucosinolate profile: aliphatic glucosinolate levels drop off more rapidly, consistent with the early senescence of the mutant, and the levels of two indole glucosinolates are uniformly low. In TU8 seeds, four long-chain aliphatic glucosinolates have significantly increased levels, whereas the indolyl-3-methyl glucosinolate level is significantly reduced relative to wild type. Genetic mapping and DNA sequencing identified the TU8 mutation as *tfl2-6*, a new allele of *TERMINAL FLOWER2* (*TFL2*), the only *Arabidopsis* homolog of animal *HETEROCHROMATIN PROTEIN1* (*HP1*). *TU8* (*tfl2-6*) has other previously identified *tfl2* phenotypes, including an early transition to flowering, altered meristem structure, and stunted leaves. Analysis of two additional alleles, *tfl2-1* and *tfl2-2*, showed glucosinolate profiles similar to those of line TU8 (*tfl2-6*).

**Abbreviations:** ABRC, *Arabidopsis* Biological Resource Center; 4BOB, 4-benzoyloxybutyl glucosinolate; 3BOP, 3-benzoyloxypropyl glucosinolate; Col-0, Columbia; EMS, ethylmethanesulfonate; GSL, glucosinolate; 4HB, 4-hydroxybutyl glucosinolate; 3HP, 3-hydroxypropyl glucosinolate; IAA, indole acetic acid; I3M, indolyl-3-methyl glucosinolate; IAOx, indole-3-acetaldoxime; *Ler*, Landsberg *erecta*; 1MI3M, 1-methoxy-indolyl-3-methyl glucosinolate; 4MI3M, 4-methoxy-indolyl-3-methyl glucosinolate; 4MSB, 4-methylsulfinylbutyl glucosinolate; 7MSH, 7-methylsulfinylheptyl glucosinolate; 8MSO, 8-methylsulfinyloctyl glucosinolate; 3MSP, 3-methylsulfinylpropyl glucosinolate; 4MTB, 4-methylthiobutyl glucosinolate; 7MTH, 7-methylthioheptyl glucosinolate; 8MTO, 8-methylthiooctyl glucosinolate; 5MTP, 5-methylthiopentyl glucosinolate; TILLING, targeted induced localized lesions in genomes

### Introduction

Glucosinolates are a class of amino acid-derived secondary metabolites that are found almost exclusively in the Brassicaceae and a few other

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species in the order Capparales (Bones and Rossiter, 1996). Together with the enzyme myrosinase, glucosinolates comprise a two-component defensive system that is active against both herbivores and microbial pathogens (Raybould and Moyes, 2001). In an undamaged plant, glucosinolates and myrosinase are contained in separate plant cells or in separate intracellular compartments (Koroleva *et al.*, 2000; Husebye *et al.*, 2002). Upon tissue damage by an herbivore, the glucosinolates come into contact with myrosinase and are hydrolyzed. Further degradation results in the production of a variety of isothiocyanates, thiocyanates, nitriles, oxazolidine-2-thiones, and epithionitriles (Wittstock and Halkier, 2002). Although there is evidence that some glucosinolates are insect-repellent in the absence of cleavage by myrosinase (Raybould and Moyes, 2001), it is thought that the secondary breakdown products have the primary defensive function. However, for some insects that are specialized for feeding on Brassicaceae, glucosinolates and their breakdown products are attractants or feeding stimulants (Pivnick *et al.*, 1994; Giamoustaris and Mithen, 1995; Smart *et al.*, 1997; Müller *et al.*, 2001).

In *Arabidopsis*, most glucosinolates are derived from the amino acids methionine, tryptophan, and phenylalanine. More recently, leucine has also been proposed as an *Arabidopsis* glucosinolate precursor (Reichelt *et al.*, 2002). Chain-elongation and secondary modification of these amino acid side chains result in at least 37 glucosinolates being detected in different *Arabidopsis* accessions (Kliebenstein *et al.*, 2001b; Reichelt *et al.*, 2002). A majority of the biosynthetic genes involved in the production of *Arabidopsis* aliphatic glucosinolates have been identified (Wittstock and Halkier, 2002). Aliphatic glucosinolates, derived from methionine, are formed through a series of chain elongation steps (Campos *et al.*, 2000; Kroymann *et al.*, 2001). Amino acids and chain-elongated products are converted to aldoximes by cytochromes P450 of the CYP79 family in the production of glucosinolates. Of the seven CYP79 genes in the *Arabidopsis* genome, five were shown to be involved in glucosinolate biosynthesis (Hull *et al.*, 2000; Mikkelsen *et al.*, 2000; Wittstock and Halkier, 2000; Hansen *et al.*, 2001; Chen *et al.*, 2003). CYP83B1 catalyzes the first committed step in the synthesis of indole glucosinolates from indole-3-acetaldoxime (IAOx) (Bak and Feyereisen, 2001; Bak *et al.*, 2001). Finally, genes responsible for alkyl-

ation and hydroxylation of amino acid side chains after synthesis of the core glucosinolate structure have been discovered through comparison of *Arabidopsis* accessions (Kliebenstein *et al.*, 2001c).

The biosynthesis of indole glucosinolates and indole-3-acetic acid (IAA) are closely connected, with IAOx serving as a precursor for the biosynthesis of both molecules. In addition, there is evidence that indole glucosinolates themselves can serve as precursors for IAA biosynthesis in a parallel pathway (Ljung *et al.*, 2002). Ectopic overexpression of CYP79B2, which catalyzes the conversion of tryptophan to IAOx, increases the levels of both IAA and indole glucosinolates (Mikkelsen *et al.*, 2000; Zhao *et al.*, 2002). Loss of function mutations in CYP83B1 result in increased IAA levels, and overexpression of this protein results in an IAA deficit, presumably by altering the amount of IAOx available for IAA biosynthesis (Bak and Feyereisen, 2001; Bak *et al.*, 2001). Thus, regulation of indole glucosinolate biosynthesis in crucifers may have developmental implications.

In contrast to the well-documented biosynthetic pathways, relatively little is known about the genetic and environmental regulation of glucosinolate accumulation. Quantitative and qualitative differences in glucosinolate content are found in various *Arabidopsis* accessions. Although some of this variation is due to the presence or absence of particular biosynthetic genes, other natural variation is likely due to differences in regulatory genes (Kliebenstein *et al.*, 2001a, b, c). In addition, levels of leaf glucosinolates vary considerably during the course of plant development (Petersen *et al.*, 2002; Brown *et al.*, 2003). Not surprisingly, *Arabidopsis* glucosinolate production is induced in response to insect feeding (Kim and Jander, unpublished) or mechanical damage (Mikkelsen *et al.*, 2003). Analysis of *Arabidopsis* signal transduction mutants and treatment of plants with the inducers methyl jasmonate and 2,6-dichloro-isonicotinic acid showed that the jasmonate- and salicylate-mediated defense pathways are involved in the induction of different classes of glucosinolates (Kliebenstein *et al.*, 2002; Mikkelsen *et al.*, 2003).

Haughn *et al.* (1991) isolated six mutants with altered leaf glucosinolate levels in an HPLC-based screen of leaves from ethylmethanesulfonate (EMS) mutagenized *Arabidopsis* Columbia (Col-0) plants. One line, called TU8, was reported to have

an unusual phenotype: reduced levels of both aliphatic and aromatic leaf glucosinolates. This reduced glucosinolate content co-segregated with early flowering and stunted rosette leaf phenotypes. Later work by others showed that the TU8 mutant is highly pleiotropic, with shorter stature, increased branching, altered levels of IAA, increased indole glucosinolate levels in roots, increased resistance to the fungal pathogen *Plasmodiophora brassicae* (clubroot), lower thermotolerance, and reduced expression of cytosolic Hsp90 (Ludwig-Müller *et al.*, 1999, 2000). All of these diverse phenotypes appeared to be caused by a single mutation.

In this manuscript we report a detailed analysis of the leaf and seed glucosinolate content of the TU8 mutant. We used map-based cloning to identify a mutation in the *TERMINAL FLOWER 2 (TFL2)* gene, which encodes a chromodomain protein that was previously identified due to its effects on flowering time. Finally, we established the identity of this gene by showing that two independently isolated alleles have the same effects on glucosinolate biosynthesis as TU8.

## Material and methods

### *Plants and growth conditions*

The TU8 mutant was obtained from G. Haughn, University of British Columbia (Haughn *et al.*, 1991), and has been submitted to the ABRC (Arabidopsis Biological Resource Center, <http://www.arabidopsis.org/abrc/>, stock number CS6397). Col-0 wild type, Landsberg *erecta* (Ler), Col-0 *tfl2-1*, and Col-0 *tfl2-2* were obtained from the ABRC. Arabidopsis plants were grown in Conviron (Winnipeg, Canada) growth chambers in standard nursery flats (approximately 20 by 40 cm) using Metromix 200 potting soil (Scotts, Marysville, OH) without additional fertilizer at 23 °C under cool white fluorescent light with an intensity of 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density (PPFD) and a 16:8 day:night photoperiod.

### *Glucosinolate analysis*

Arabidopsis seed and leaf glucosinolate analysis was performed by adapting a previously published protocol (Hogge *et al.*, 1988) to 96-well microtiter plates. Seed extracts were prepared by grinding

2 mg dry seeds with one 3 mm steel ball bearing for 1 min in a 1.2 ml tube on a Harbil model 5G-HD paint shaker (Harbil, Wheeling, IL), adding 290  $\mu\text{l}$  of 60% methanol and 10  $\mu\text{l}$  5 mM sinalbin standard (4-hydroxybenzyl glucosinolate, a gift from A. Renwick), and grinding again for 1 min. For leaf glucosinolate assays, the two largest leaves from each plant were collected for glucosinolate analysis, 750  $\mu\text{l}$  cold 60% methanol were added to 40–130 mg fresh leaf material in a 1.2 ml tube, and samples were frozen immediately at  $-80$  °C. Plants from which leaves were harvested were not used again for subsequent experiments. Samples from all time points of a particular experiment were stored at  $-80$  °C and extracted simultaneously. Sinalbin (10  $\mu\text{l}$  of 1 mM) was added to each tube of leaf material as an internal standard before extraction and the sample was then vortexed briefly.

Seed and leaf extracts were heated for 10 min in a 75 °C water bath to extract glucosinolates, then cooled down to room temperature. DEAE Sephadex A-25 (Amersham, Buckinghamshire, UK) columns were prepared by adding 45  $\mu\text{l}$  (dry volume) resin to each well of 96-well, 2 ml well volume microtiter plates, which had 0.45  $\mu\text{m}$  pore size hydrophilic PVDF filter-bottoms (Whatman, Maidstone, UK). The resin was swelled with water and Arabidopsis extracts (400  $\mu\text{l}$  for leaf or 100  $\mu\text{l}$  seed) were added to each well. Extracts were incubated on the columns for 15 min, liquid was pulled through using a vacuum manifold (Qiagen, Valencia, CA), and columns were rinsed once with 750  $\mu\text{l}$  60% methanol and twice with 950  $\mu\text{l}$  water. Aryl sulfatase (Sigma, St. Louis, MO) solution (150  $\mu\text{l}$  of 0.004 g/ml) was then added to each well of the plate and the plate left overnight in the dark at room temperature. To elute desulfoglucosinolates, 500  $\mu\text{l}$  water was added to each column and the column plate was centrifuged at  $50 \times g$  for 15 min with a collection plate underneath. Desulfoglucosinolates were stored at  $-80$  °C.

Samples were loaded onto a LiChrospher RP-18,  $250 \times 4.6$  mm, 5  $\mu\text{m}$ , MetaChem column with a MetaGuard LiChrosphere RP-18, 4.6 mm, 5  $\mu\text{m}$ , end-capped guard column (Varian, Torrance, CA) using a Waters 2700 Sample Manager and a Waters 2690 Separations Module (Waters, Bedford, MA). Mobile phases were A, water and B, 90% acetonitrile. Column linear gradients for leaf samples were: 0–6 min, 98% A, 2% B; 6–8 min

94% A, 6% B; 8–18 min 92% A, 8% B; 18–23 min 77% A, 23% B, 23–30 min 69% A, 31% B, 30–32 min 55% A, 45% B; 32–37 min 100% B; 37–39 min hold 100% B; 39–50 min 98% A, 2% B, with a flow rate of 1 ml/min. Column linear gradients for seed samples were: 0–8 min, 98% A, 2% B; 8–10 min 94% A, 6% B; 10–35 min 92% A, 8% B; 35–37 min 57% A, 43% B; 37–41 min 100% B; 41–43 min hold 100% B; 43–55 min 98% A, 2% B; with a flow rate of 1 ml/min.

Desulfoglucosinolates were detected using a Waters 996 Photodiode Array Detector at 229 nm wavelength, and masses of the parent ions were confirmed using a Micromass Platform LC mass spectrometer (Waters, Bedford, MA).

Desulfated glucosinolate concentrations were calculated relative to sinalbin using published (Brown *et al.*, 2003) response factors (molar extinction at 229 nm relative to sinigrin, 2-propenyl glucosinolate, set at 1.0): sinalbin (0.4), 3HP (2.1), 4HB (1.4), 3MSP (1.2), 4MSB (0.9), 4MTB (0.9), 8MSO (1.1), 7MTH (1.0), 8MTO (1.1), 13M (0.3), 4MI3M (0.3), 1MI3M (0.2), 3BOP (0.4) and 4BOB (0.3). Response factors for 7MSH and 5MTP were estimated to be 1.0 based on response factors of glucosinolates with similar structures (Brown *et al.*, 2003).

#### *Crosses, genetic analysis, and map-based cloning*

Crosses with TU8 plants were performed using standard methods (Weigel and Glazebrook, 2002). For crosses to wild-type Col-0 and *Ler*, TU8 was used as the male parent. For crosses with *tfl2-1* and *tfl2-2*, TU8 was used as the female parent.

Mapping of the TU8 mutation followed a previously outlined protocol (Jander *et al.*, 2002) using markers from the Cereon/Monsanto Arabidopsis Polymorphism Collection (<http://www.arabidopsis.org/Cereon>) and one previously unpublished marker. F<sub>2</sub> seeds were collected from F<sub>1</sub> plants and were used to generate an initial mapping population of approximately 600 F<sub>2</sub> plants. Among these plants, those with the characteristic dwarf phenotype (homozygous for the TU8 mutation) were identified and genotyped using five SSR markers on each of the five chromosomes of Arabidopsis. DNA surrounding the markers was amplified by PCR and the genotype at the site of the mutation was determined using a Li-Cor (Lincoln, Nebraska) sequencing apparatus.

Linkage analysis was used to identify markers that flank the TU8 mutation.

For fine-scale mapping, an additional 4000 TU8 x *Ler* F<sub>2</sub> seeds were planted and approximately 1000 lines homozygous for the TU8 mutation were chosen for analysis. Lines that showed genetic recombination between the previously identified flanking markers were used to narrow down the mutation site with SNP markers that were genotyped by PCR amplification and DNA sequencing. To identify the TU8 mutation, DNA was amplified from leaf samples and sequenced as described previously (Jander *et al.*, 2003).

#### *Data analysis*

Statistical analysis was performed with Stata for Macintosh, version 6.0 (StataCorp, College Station, TX) and Microsoft Excel (Microsoft, Redmond, WA).

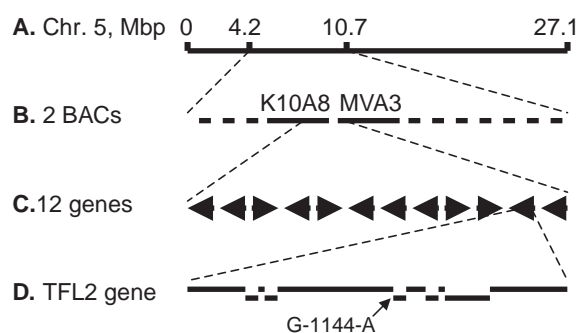
## **Results**

#### *Verification of TU8 mutant phenotypes*

The TU8 mutant was backcrossed once to wild-type Col-0, and the identity of the mutation was confirmed by assessing some of the previously reported phenotypes (Haughn *et al.*, 1991; Ludwig-Müller *et al.*, 1999, 2000). Leaf glucosinolate content, the phenotype assayed in the original mutant screen, was reduced compared to that of wild-type plants. Also consistent with published reports, we observed short stature, increased branching, stunted rosette leaves, flower initiation 8 or 9 days early than wild-type Col-0 under long-day conditions, and temperature sensitivity.

#### *Map-based cloning of the TU8 mutation*

As shown in Figure 1, the genetic basis of the TU8 mutation was determined using standard Arabidopsis map-based cloning methods (Jander *et al.*, 2002). One of the two closest flanking markers is CER440765 in the Cereon/Monsanto Arabidopsis Polymorphism Collection (<http://www.arabidopsis.org/Cereon/index.html>) on the chromosome 5 P1 clone MVA3 (AB006706). The other nearest flanking marker is an unpub-



**Figure 1.** Overview of map-based gene identification for the TU8 mutation. A: simple sequence repeat (SSR) markers were used to narrow the position to a 6.5 Mbp interval on chromosome 5; B, C: single nucleotide polymorphism (SNP) markers were used in mapping to a 41 kbp region with 12 predicted genes on two BACs (K10A8, gi:9755777; and MVA3, gi:2351071); and D: DNA sequencing identified a G-1144-A change in the *TFL2* gene. Alternating bars represent predicted exons (raised bars) and introns (lower bars) of *TFL2*, with the protein N-terminus on the right.

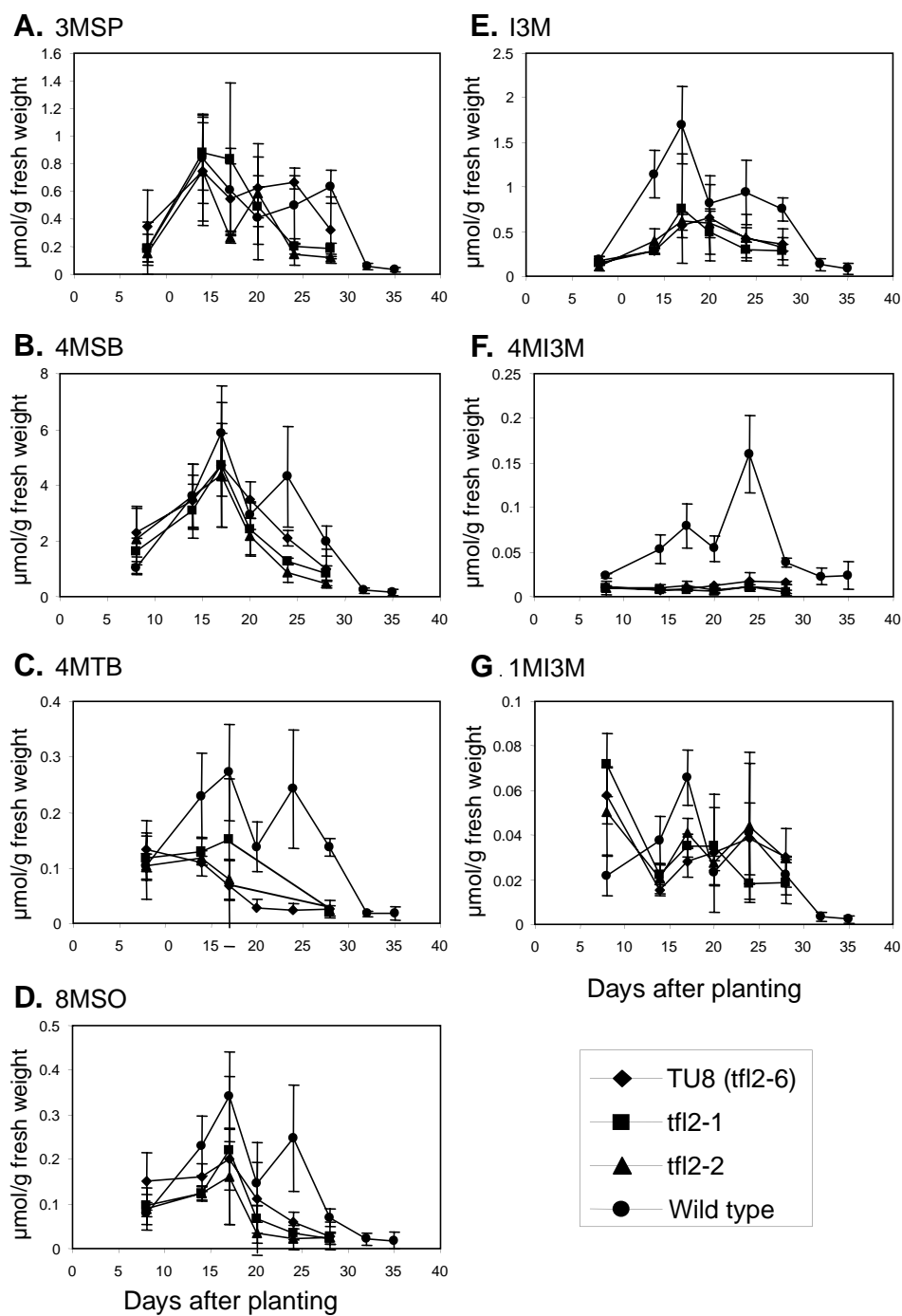
lished A/G Col-0/*Ler* change at bp 20664 on chromosome 5 BAC clone K10A8 (AL391151). The 41 kbp of chromosomal DNA between the final flanking markers were sequenced from the TU8 mutant, and a G to A difference at bp 10789 of BAC MVA3 was identified. This is consistent with the G:C to A:T transitions that are typically induced by EMS mutagenesis (Kreig, 1963; Jander *et al.*, 2003; Till *et al.*, 2003). The identified mutation is at bp 1144 in the 5' splice site of the third intron of a gene (AB073490; AT5G17690) that was previously identified as *TERMINAL FLOWER 2 (TFL2)* (Larsson *et al.*, 1998; Kotake *et al.*, 2003) and *LIKE HETEROCHROMATIN PROTEIN 1 (LHP1)* (Gaudin *et al.*, 2001).

#### *Leaf and seed glucosinolate phenotypes of TU8, tfl2-1, and tfl2-2 mutants*

To confirm the identity of the TU8 mutation, we compared leaf and seed glucosinolate levels of Col-0 wild type, TU8 mutant, and plants carrying two additional *tfl2* alleles (Larsson *et al.*, 1998) that were obtained from the ABRC (*tfl2-1*, accession CS3796; and *tfl2-2*, accession CS3797). Rosette leaf glucosinolate levels were measured at three or six-day intervals, beginning at day eight after planting, and ending when the leaves were becoming senescent, on day 28 for the mutants and day 35 for the wild-type plants (Figure 2). Wild-

type glucosinolate profiles were similar to what has been reported previously, with a peak at the onset of flowering, and low levels in leaves undergoing senescence (Petersen *et al.*, 2002; Brown *et al.*, 2003). The aliphatic glucosinolates, 3MSP, 4MSB, 4MTB, and 8MSO (Figure 2A–D) had a similar profile in the mutants, but dropped off more rapidly, consistent with the earlier flowering and leaf senescence of these plants. Two indole glucosinolates, I3M and 4MI3M (Figure 2E, F), had uniformly low levels in all three mutants throughout development. 1MI3M levels in mutant plants were higher than wild type on day eight, but later dropped to wild-type levels (Figure 2G). Total aliphatic glucosinolate content (3MSP + 4MSB + 4MTB + 8MSO) was significantly lower in *tfl2* mutant than in wild type plants on days 24 and 28. Total indole glucosinolate levels (I3M + 1MI3M + 4MI3M) were lower in mutant than in wild type plants on days 14 through 28, though the decrease was not significant at the 95% confidence level on day 20. An independent experiment produced results similar to those in Figure 2 (data not shown). Additional glucosinolates are present at low levels in *Arabidopsis* leaves (Brown *et al.*, 2003), but were not included in this analysis because the HPLC quantitation was less reliable.

In contrast to the initial report of the TU8 mutant (Haughn *et al.*, 1991), we also measured significantly altered seed glucosinolate profiles in TU8, *tfl2-1* and *tfl2-2* mutants (Table 1). Seeds were collected from three independently grown sets of mutant and wild-type plants. The first column of Table 1 shows wild-type seed glucosinolate levels averaged across all three experiments. Environmental factors beyond our control caused a two-fold range in both mutant and wild type mean glucosinolate levels in the three experiments, but the relative levels of mutant and wild type were similar in each run. Therefore, to facilitate comparison of mutant and wild-type data, mutant glucosinolate levels were expressed as a percentage of the wild-type mean for each independent run of the experiment. Relative to wild type, TU8 had significant increases in long-chain aliphatic glucosinolates (7MSH, 8MSO, 7MTH, and 8MTO) and a significant decrease in I3M, the only abundant indole glucosinolate in Col-0 seeds. These results were largely the same with the other two mutants, though the increase in 8MTO for *tfl2-1* and the



**Figure 2.** Developmental variation in the levels of four aliphatic (A–D; 3MSP=3-methylsulfinylpropyl-; 4MSB=4-methylsulfinylbutyl-; 4MTB=4-methylthiobutyl-; 8MSO=8-methylsulfinyloctyl-) and three aromatic (E–G; I3M=indolyl-3-methyl-; 1MI3M=1-methoxy-indolyl-3-methyl-; 4MI3M=4-methoxy-indolyl-3-methyl-) leaf glucosinolates in Col-0 wild type (circles), TU8 (diamonds), *tf12-1* (squares), and *tf12-2* (triangles). The *x*-axis indicates days after planting. Data points represent the mean and standard deviation of, in most cases, three or four samples. 4MTB concentration was not determined for *tf12-1* and *tf12-2* on days 20 and 24 due to ambiguous peaks.

Table 1. Seed glucosinolate content of Col-0 wild type and *tfl2* mutants.

Glucosinolate <sup>a</sup>	wild type $\mu\text{mol/g}^b$	TU8 ( <i>tfl2-6</i> )		<i>tfl2-1</i>		<i>tfl2-2</i>		
		Mean <sup>c</sup> (%)	Error <sup>d</sup> (%)	Mean <sup>c</sup> (%)	Error <sup>d</sup> (%)	Mean <sup>c</sup> (%)	Error <sup>d</sup> (%)	
3HP	2.0 $\pm$ 1.3	149	23	215	41	169	26	
4HB	10.6 $\pm$ 6.5	102	14	141	17	153	21	
4MSB	5.4 $\pm$ 1.9	103	18	175	41	118	22	
7MSH	2.8 $\pm$ 0.8	164	13	183	20	192	23	**
4MTB	22.7 $\pm$ 7.7	125	13	151	19	167	20	
8MSO	7.9 $\pm$ 3.8	225	26	243	33	260	38	**
5MTP	3.3 $\pm$ 0.7	94	6	102	6	129	12	
7MTH	4.7 $\pm$ 0.5	151	7	151	7	180	15	**
8MTO	3.3 $\pm$ 1.3	186	17	170	17	206	18	*
Aliphatic GSL	74 $\pm$ 22	111	9	123	12	147	18	
3BOP	2.9 $\pm$ 0.9	143	11	150	12	199	15	**
4BOB	3.9 $\pm$ 2.0	121	21	164	31	184	25	
Benzoyl GSL	6.8 $\pm$ 2.9	131	16	158	22	190	18	
I3M	2.6 $\pm$ 1.3	33	9	51	10	75	11	**
Total GSL	84 $\pm$ 21	110	8	124	10	148	16	

<sup>a</sup> 3HP = 3-hydroxypropyl-, 4HB = 4-hydroxybutyl-, 4MSB = 4-methylsulfinylbutyl-, 7MSH = 7-methylsulfinylheptyl-, 4MTB = 4-methylthiobutyl-, 8MSO = 8-methylsulfinyloctyl-, 5MTP = 5-methylthiopentyl-, 7MTH = 7-methylthioheptyl-, 8MTO = 8-methylthiooctyl-, 3BOP = 3-benzoyloxypropyl-, 4BOB = 4-benzoyloxybutyl-, and I3M = indolyl-3-methyl glucosinolate.

<sup>b</sup> Mean and standard deviation of seed glucosinolate data from three independent experiments,  $N = 20$  (3HP, 4HB, 4MSB, 7MSH, 4MTB, 8MSO, I3M) or  $N = 10$  (5MTP, 3BOP, 4BOB, 7MTH, 8MTO, aliphatic GSL, benzoyl GSL, total GSL).

<sup>c</sup> Mean mutant seed glucosinolate content as a percentage of the wild-type mean in each of three experiments,  $N = 13$  (3HP, 4HB, 4MSB, 7MSH, 4MTB, 8MSO, I3M) or  $N = 9$  (5MTP, 3BOP, 4BOB, 7MTH, 8MTO, aliphatic GSL, benzoyl GSL, total GSL).

<sup>d</sup> Standard error of the difference of the mutant and wild-type means.

\*  $P < 0.05$ , \*\*  $P < 0.01$ , Mann-Whitney U-test with Bonferroni correction for multiple pair-wise comparisons.

decrease in I3M for *tfl2-2* were not significant at the 95% confidence level. In addition, an observed increase in 3BOP was only significant for *tfl2-2*. Although there were significant differences in the levels of individual glucosinolates, the total seed glucosinolate content was not significantly different between *tfl2* mutant and wild-type plants.

#### Flowering phenotypes of TU8, *tfl2-1*, and *tfl2-2* mutants

The inflorescence development of TU8, *tfl2-1*, and *tfl2-2* are indistinguishable from one another (Figures 3 and 4). All three mutants started producing flower stalks eight days before wild-type plants. Neither shoot height nor shoot number was significantly different among the three mutants at any stage of development. At the end of the observation period, all three mutants had produced significantly more (Figure 3A), but shorter (Figure 3B) shoots than wild-type plants, similar to observations of TU8 alone made by Ludwig-

Müller *et al.* (1999). The numbers of rosette leaves one day before the onset of flowering in this experiment (day 11) were not significantly different among the mutants: TU8, 5.7  $\pm$  0.6; *tfl2-1*, 5.9  $\pm$  0.4; and *tfl2-2*, 5.7  $\pm$  0.5. Wild-type plants had significantly more rosette leaves one day before the onset of flowering (8.2  $\pm$  0.7 on day 19) than any of the mutants, an effect that is similar to what has been observed previously for *tfl2-1* and *tfl2-2* mutant plants under long-day growth conditions (Kotake *et al.*, 2003, Takada and Goto, 2003). F<sub>1</sub> plants generated by crossing TU8 to *tfl2-1* and *tfl2-2* have flowering time and shoot morphology phenotypes that are similar to the three parents (Figure 4), confirming that these recessive mutations are allelic.

#### Evidence that strong alleles are required for changes in morphology and secondary chemistry

It is striking that all known alleles of *TFL2/LHP1*/TU8 are predicted to have similarly severe mutant

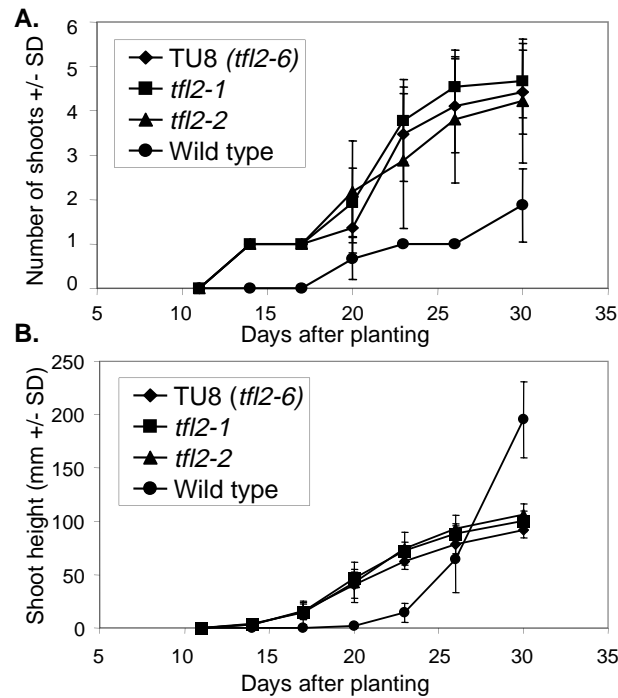


Figure 3. Comparison of number of shoots (A), and height of the tallest shoot (B) of *tfl2* mutant and wild-type plants during the course of development. Data points represent mean and standard deviation of 45–72 samples.

defects. For instance, it is likely that the TU8 mutation would result in mis-splicing and dramatic reduction of TFL2 protein, because comparison of genomic and cDNA sequences in GenBank (gi:2351071 and gi:15625406) confirms that this is an active splice site. Other published alleles of *TFL2* are also predicted to be knockout mutations: *tfl2-1* and *lhp1-2* create stop codons, *tfl2-2* has a 1.3 kb promoter deletion, *tfl2-3* contains a T-DNA insertion, and *lhp1-1* has a T-DNA

insertion associated with a 1.2 kb deletion (Gaudin *et al.*, 2001; Kotake *et al.*, 2003). To test the hypothesis that leaky alleles do not share the syndrome of morphological aberration and altered glucosinolates, additional *tfl2* mutants isolated in the Arabidopsis TILLING (Targeted Induced Localized Lesions IN Genomes) Project (Till *et al.*, 2003) were obtained from the Arabidopsis Biological Resource Center (accessions CS89111, CS90157, CS90288, CS90486, CS91146, CS91149,

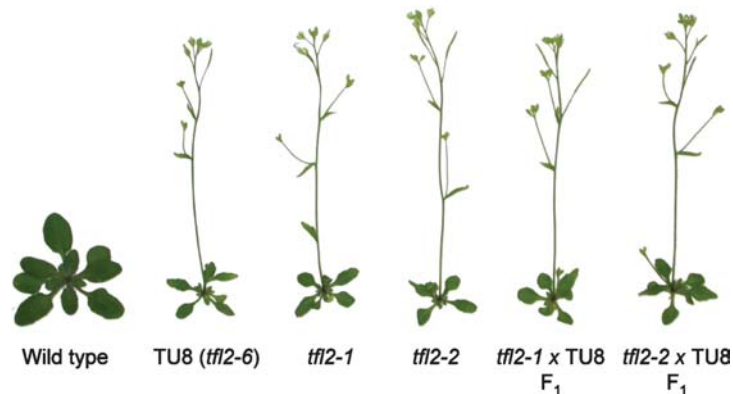


Figure 4. Photographs of Col wild type, TU8, *tfl2-1*, *tfl2-2*, and F<sub>1</sub> progeny from crosses between mutants, 20 days after planting.

CS91847, CS91850, CS91951, CS92965, CS93252, and CS93530). Four of these alleles are predicted not to alter the amino acid sequence of the protein and eight are missense mutations. In contrast to the severe alleles in TU8, *tfl2-1*, and *tfl2-2*, the TILLING missense mutations might be expected to have a more modest phenotype. Indeed, analysis of leaf glucosinolates in the TILLING lines did not show any significant reduction relative to wild-type levels (data not shown). In addition, none of the TILLING lines showed the characteristic early flowering and stunted leaf phenotypes seen in TU8 and other *TFL2* alleles (Larsson *et al.*, 1998; Gaudin *et al.*, 2001; Kotake *et al.*, 2003). We conclude that a severe mutation, or perhaps a complete knockout of *TFL2* activity, is needed to observe these phenotypes.

## Discussion

Our results show that a lesion in *TFL2* is the genetic basis of the TU8 mutation, which alters glucosinolate levels, flowering time, and other phenotypes in Arabidopsis. Genetic mapping, followed by sequencing of *TFL2* from line TU8 identified a splice-site mutation in the third intron of this gene (Figure 1). Moreover, there was no complementation in F<sub>1</sub> plants from crosses between TU8 and *tfl2-1* or *tfl2-2* (Figure 4), indicating that these recessive mutations are in the same gene and have similarly severe effects. Therefore, we propose that the TU8 mutation should be called *tfl2-6*, a new allele of *TFL2*.

The different developmental rates of the TU8 mutant and Col-0 wild-type plants complicate the accurate comparison of glucosinolate levels between these two lines (Figure 2). In contrast to our results, Haughn *et al.* (1991) found an overall reduction of glucosinolates in plants that were two to three weeks old. It is possible that line TU8 was already becoming senescent at the time that leaf samples were collected for analysis. Because leaf glucosinolate levels drop as Arabidopsis plants undergo senescence (Figure 2; Petersen *et al.*, 2002; Brown *et al.*, 2003), this could explain the decreased levels of aliphatic leaf glucosinolates that were reported for the initial isolation of the TU8 mutant. Environmental differences, which have a strong influence on glucosinolate content, are another possible explanation for the discrep-

ancies between our leaf glucosinolate measurements and those of Haughn *et al.* (1991). Mature seeds, though their glucosinolate content depends on the developmental progression of the parent plant, are themselves metabolically fairly static. Using seed glucosinolate assays, it is possible to compare mutant and wild-type plants at the same developmental stage. The seed glucosinolate data (Table 1) show that long-chain aliphatic glucosinolates (7MSH, 8MSO, 7MTH, and 8MTO) are significantly more abundant in TU8 mutant than in wild type plants. Because there are maternal effects on seed glucosinolate content (Macgrath and Mithen, 1993; Brudenell *et al.*, 1999; Chen *et al.*, 2001), these changes could be due to differences in plant development, transport of glucosinolates, or biosynthesis in developing seeds. Similar effects on aliphatic glucosinolates were observed due to the *tfl2-1* and *tfl2-2* mutations, with a few disparities that could be due to statistical variation or perhaps minor effects of other mutations in these lines.

In contrast to aliphatic glucosinolates, the effects of *tfl2* mutations on leaf indole glucosinolate content cannot be easily explained by the growth stage of the plants. Two weeks after planting, when the *tfl2* mutants had just started flowering, the levels of aliphatic glucosinolates were similar in mutant and wild type plants, and the mutants had markedly decreased levels of only the tryptophan-derived glucosinolates, I3M and 4MI3M (Figure 2E, F). In two previous reports, I3M and 1MI3M (but not 4MI3M) were induced by treatment with methyl jasmonate (Kliebenstein *et al.*, 2002; Mikkelsen *et al.*, 2003), an indication that altered glucosinolate production due to *tfl2* mutations is distinct from the jasmonate response. I3M, the only prominent indole glucosinolate in seeds, is also the only glucosinolate with significantly lower levels in seeds of *tfl2* mutants than in Col-0 wild type. In contrast, roots of TU8 mutants were reported to have higher levels of three indole glucosinolates, I3M, 4MI3M, and 1MI3M (Ludwig-Müller *et al.*, 1999). Although we did not measure root glucosinolate levels, leaves of young *tfl2* plants (day 8) had higher levels of 1MI3M than wild-type (Figure 2G).

Our data are consistent with published observations that there are strong environmental effects on both seed and leaf glucosinolate content. In three independent experiments we observed a

two-fold range in seed glucosinolate content in plants that were grown in the same growth chamber, even though the variation within an individual experiment was quite low. Factors that are difficult to control, such as the exact composition of the soil, changes in light levels over time, or the presence of microorganisms, may affect glucosinolate levels. The wild-type seed glucosinolate levels that we measured are similar to those of two other studies (Petersen *et al.*, 2002; Brown *et al.*, 2003), but higher than those of a third (Kliebenstein *et al.*, 2001b). Levels of leaf glucosinolates are even more difficult to compare between studies. The variance in leaf glucosinolates is higher than in seeds, because the growth stage of the plant, diurnal variation in glucosinolate levels, and the exact tissue that is harvested may affect the measurements. In addition, other recent reports measured glucosinolates from lyophilized tissue (Kliebenstein *et al.*, 2001b; Petersen *et al.*, 2002; Brown *et al.*, 2003), whereas we used frozen leaves.

*TFL2* encodes the Arabidopsis homolog of the yeast Swi6 protein and HETEROCHROMATIN PROTEIN 1 (HP1), which has been identified in *Drosophila* and other animals (Gaudin *et al.*, 2001; Kotake *et al.*, 2003). These influence gene regulation through the initiation and maintenance of condensed heterochromatin structure (Paro and Hogness, 1991; Eissenberg and Elgin, 2000). As such, TFL2 is a member of the chromodomain family of proteins, which is conserved in animals, plants, and yeast. TFL2 contains both the chromodomain and the chromo shadow domain that are critical for the function of *Drosophila* and mammalian HP1 homologs in gene regulation (Eissenberg and Elgin, 2000; Jones *et al.*, 2000). The punctate nuclear localization pattern of TFL2-GFP fusions (Gaudin *et al.*, 2001) is also consistent with such a role for TFL2.

Given the broad regulatory function of chromodomain proteins, it is likely that absent or reduced TFL2 protein causes altered transcription of a variety of Arabidopsis genes. Therefore, the pleiotropic phenotypes of *tfl2* mutants may be due to direct or indirect effects on multiple metabolic pathways. Gene expression analysis showed that TFL2 functions as a repressor of several floral homeotic genes (Kotake *et al.*, 2003) and counteracts the effects of CONSTANS by repressing *FLOWERING LOCUS T* (Takada and Goto, 2003), which explains the early flowering pheno-

type that is seen in *tfl2* mutants. Temperature sensitivity of the TU8 mutant may result from decreased HSP90 expression (Ludwig-Müller *et al.*, 2000).

Because the biosyntheses of IAA and indole glucosinolates are closely connected (Ljung *et al.*, 2002), it is tempting to speculate on a direct causal relationship between the reduced indole glucosinolates and altered IAA levels of the TU8 mutant. It is possible that strong *tfl2* mutations have a global effect on indole metabolism in Arabidopsis. Ludwig-Müller *et al.* (1999) showed that, compared to wild-type Col-0, the TU8 mutant has increased tryptophan levels, an earlier developmental peak in leaf IAA levels, and an earlier increase in the levels of tryptophan-oxidizing enzyme. On the other hand, if indole glucosinolates serve as a direct precursor for IAA (Ljung *et al.*, 2002; Zhao *et al.*, 2002), leaf morphology changes in *tfl2* mutants may simply be an indirect effect of altered glucosinolate levels.

Our results suggest a variety of future studies that can be used to test the above hypotheses. For instance, microarray analysis of gene expression related to IAA and indole glucosinolate biosynthesis in mutant and wild-type plants might show an effect of TFL2 protein on genes encoding enzymes of these pathways. The developmental defects of *tfl2* mutants could be alleviated by treatments such as: (1) Increasing indole glucosinolates and IAA through over-expression of CYP79B2 (Mikkelsen *et al.*, 2000; Zhao *et al.*, 2002), (2) Reducing IAA levels through over-expression of CYP83B1 (Bak and Feyereisen, 2001; Bak *et al.*, 2001), or (3) Combining *tfl2* mutations with other mutations, e.g. *yucca* (Zhao *et al.*, 2001, 2002) or *rooty* (King *et al.*, 1995), that affect IAA biosynthesis.

By demonstrating that the TU8 mutant has a lesion in *TFL2*, we have connected a new set of phenotypes to mutations in this gene: reduced leaf glucosinolates (Haughn *et al.*, 1991), altered IAA levels, altered tryptophan metabolism (Ludwig-Müller *et al.*, 1999), temperature sensitivity, reduced HSP90 expression (Ludwig-Müller *et al.*, 2000), and altered seed glucosinolate levels (this study). Whereas decreased aliphatic glucosinolate levels in *tfl2* leaves are most likely the result of altered developmental processes (early senescence), lower indole glucosinolate levels may reflect altered indole metabolism in these mutants. Further

investigations are needed to reveal the role that these metabolic pathways have in normal plant development.

### Acknowledgements

The authors wish to thank George Haughn for the TU8 mutant, Alan Renwick for the sinalbin standard, Terry Shea for DNA sequence analysis, and members of the Monsanto Company DNA sequencing group. This project was funded by the Monsanto Company, Atlantic Philanthropies, and the Boyce Thompson Institute.

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