Regulation of Early Tomato Fruit Development by the *Diageotropica* Gene[1]

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The vegetative phenotype of the auxin-resistant *diageotropica* (*dgt*) mutant of tomato (*Lycopersicon esculentum* Mill.) includes reduced gravitropic response, shortened internodes, lack of lateral roots, and retarded vascular development. Here, we report that early fruit development is also dramatically altered by the single-gene *dgt* lesion. Fruit weight, fruit set, and numbers of locules and seeds are reduced in *dgt*. In addition, time to flowering and time from anthesis to the onset of fruit ripening are increased by the *dgt* lesion, whereas ripening is normal. The *dgt* mutation appears to affect only the early stages of fruit development, irrespective of allele or genetic background. Expression of members of the *LeACS* (1-aminocyclopropane-1-carboxylic acid synthase, a key regulatory enzyme of ethylene biosynthesis) and *LeIAA* (*Aux/IAA*, auxin-responsive) gene families were quantified via real-time reverse transcriptase-polymerase chain reaction in both *dgt* and wild-type fruits, providing the first analysis of *Aux/IAA* gene expression in fruit. The *dgt* lesion affects the expression of only certain members of both the *LeACS* and *LeIAA* multigene families. Different subsets of *LeIAA* gene family members are affected by the *dgt* mutation in fruits and hypocotyls, indicating that the DGT gene product functions in a developmentally specific manner. The differential expression of subsets of *LeIAA* and *LeACS* gene family members as well as the alterations in *dgt* fruit morphology and growth suggest that the early stages of fruit development in tomato are regulated, at least in part, by auxin- and ethylene-mediated gene expression.

The onset of ovary development into fruit (fruit set) and fruit development are usually triggered by signals from pollination and fertilization. Fertilization-independent fruit set can also occur either naturally in parthenocarpic fruits (genetic parthenocarpy) or by induction via exogenous application of auxin or GAs to flowers. Reproductive processes in fleshy fruits have been perhaps best studied in tomato (*Lycopersicon esculentum* Mill.; Gillaspy et al., 1993; Giovannoni, 2001), and here we apply the availability of an auxin-resistant mutant of tomato to further elucidate the biochemical, genetic, and molecular mechanisms that regulate fruit set and the early stages of fruit development.

Artificial induction via auxin has long been used to study parthenocarpy in tomato (Gustafson, 1937). Application of auxin transport inhibitors that block export of auxins from the ovary also stimulates the development of parthenocarpic fruits (Beyer and Quebedeaux, 1974), an observation that is consistent with reports of higher levels of auxins in ovaries of parthenocarpic tomato fruits (Mapelli et al., 1978; Mapelli and Lombardi, 1982).

Auxins are also involved in cell expansion in fruit tissues. During tomato fruit development, two peaks in auxin content occur (Gillaspy et al., 1993). The first auxin peak occurs 10 d after anthesis, coinciding with the beginning of cell expansion. The second auxin peak appears later and coincides with the final phase of embryo development. In non-parthenocarpic tomato varieties, the number of seeds affects final fruit size (Varga and Bruinsma, 1986). Thus, embryo-synthesized auxin could be the source for the second auxin peak (Hocher et al., 1992). In accordance, in parthenocarpic fruits, this second peak is not detected and fruits are correspondingly smaller (Mapelli et al., 1978).

It is likely that auxin regulation of fruit development involves gene expression. Auxin induces the expression of several gene families, including the *SAUR* (*s*mall *a*uxin *u*p-regulated *R*NA), *GH3*, and *Aux/IAA* genes (Guilfoyle, 1998). The *Aux/IAA* genes constitute a family of early auxin response genes (Abel and Theologis, 1996) encoding proteins that contain nuclear localization signals and have short half-lives (Abel et al., 1994; Oeller and Theologis, 1995). The ability of Aux/IAA family members to form homo- and heterodimers, as well as heterodimers with DNA-binding auxin response factors, supports their role as regulators of auxin responses (for review, see Reed, 2001). In Arabidopsis, 29 *Aux/IAA* genes have been identified (Reed, 2001), some of which show differences in gene expression kinetics, tissue specificity, and responsiveness to auxin induction (Abel et al., 1995; Abel and Theologis, 1996; Kim et al., 1997). Characterization of mutant phenotypes for nine of the Arabidopsis *Aux/IAA* genes has provided functional evidence for the importance of *Aux/IAA* genes as regulators of various auxin responses

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(Timpte et al., 1992; Kim et al., 1996; Leyser et al., 1996; Reed et al., 1998; Rouse et al., 1998; Hamann et al., 1999; Tian and Reed, 1999; Nagpal et al., 2000; Reed, 2001; Rogg et al., 2001).

Several *Aux/IAA Arabidopsis* mutants also exhibit reproductive alterations as part of their phenotypes. The *axr2-1* mutant has short inflorescences because of reduced cell length and cell number (Timpte et al., 1992). In contrast, the single unbranched inflorescence of *axr3* plants is shorter than wild type because of reduced internode number (Leyser et al., 1996). The *axr3* mutant also exhibits reduced seed set compared with wild-type plants (Leyser et al., 1996). Similarly, the *iaa28-1* mutant has a lower seed yield, smaller siliques, and shorter inflorescence internodes (Rogg et al., 2001), whereas *shy2-2* mutants flower early (Reed et al., 1998). Eleven members of the *Aux/IAA* gene family are expressed in tomato vegetative tissues (Nebenführ et al., 2000), but whether any of these genes influence tomato fruit development is unknown.

The involvement of ethylene in the ripening stage of tomato fruit is well documented (Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992; Lincoln et al., 1993). However, the importance of ethylene in regulating early stages of tomato fruit growth has only recently been examined (Nakatsuka et al., 1998; Barry et al., 2000). The enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) catalyzes the first regulatory step in the ethylene biosynthesis pathway, conversion of *S*-adenosyl-L-Met into ACC, whereas ACC oxidase (ACO) catalyzes the final step, conversion of ACC into ethylene (Yang and Hoffman, 1984; Kende, 1993). Both ACS and ACO are encoded by multigene families (Fluhr and Mattoo, 1996). The eight tomato *ACS* (*LeACS*) genes characterized so far have differences in their tissue-specific expression patterns, developmental control, and kinetics of ethylene induction (Van der Straeten et al., 1990; Olson et al., 1991, 1995; Rottmann et al., 1991; Yip et al., 1992; Lincoln et al., 1993; Spanu et al., 1993; Terai, 1993; Oetiker at al., 1997; Nakatsuka et al., 1998; Shiu et al., 1998) but it is not yet known how or if they interact with auxin to regulate fruit development.

The auxin-resistant *dgt* (*diageotropica*) mutant of tomato provides a tool to further investigate the interactions between auxin and ethylene in regulating several aspects of plant development. Plants that are homozygous for any of three independent alleles of *dgt* result in the same pleiotropic phenotype, which includes: reduced apical dominance and gravitropic response, hyponastic leaves, retarded vascular development, high levels of anthocyanin and chlorophyll, and lack of lateral roots (Zobel, 1973, 1974). Although endogenous levels of IAA are the same in both *dgt* and wild-type shoot apices (Fujino et al., 1988b), *dgt* hypocotyl segments do not elongate or produce ethylene in response to exogenously applied auxin (Kelly and Bradford, 1986). Roots of the *dgt* mutant are more resistant to growth inhibition by exogenously applied IAA, auxin transport inhibitors, and ethylene than wild-type roots (Muday et al., 1995). Very low ethylene concentrations can restore the reduced gravitropic response of *dgt* to wild-type levels but not with wild-type kinetics (Madlung et al., 1999).

In hypocotyls, the *dgt* mutation reduces auxin-induced expression of a subset of auxin-regulated genes such as *LeSAUR* and the *LeIAA5, 8, 10*, and *11* members of the tomato *Aux/IAA* gene family. However, the *dgt* mutation has no effect on the expression of other auxin-inducible genes such as *Lepar* and several other members of the *LeIAA* gene family (Mito and Bennett, 1995; Nebenführ et al., 2000). The expression of two auxin-regulated *ACS* (*LeACS*) gene family members is also reduced in response to applied auxin in *dgt*, but not wild-type seedlings, whereas that of another auxin-regulated *LeACS* gene is not (Coenen and Lomax, 2003). The means by which subsets of auxin- and ethylene-regulated genes are affected by the *dgt* mutation has not yet been determined.

Although the pleiotropic effects of the *dgt* mutation on a variety of auxin responses during vegetative development are well studied, the only published reports of *dgt* reproductive development state briefly that it is normal (Fujino et al., 1988a; Ludford, 1995). Here, we document profound differences in fruit development in *dgt* versus wild-type plants. The expression of a subset of *LeACS* and *LeIAA* gene family members is also altered in *dgt* fruits. The observed changes are specific to early fruit development and different from those observed in vegetative tissues, indicating developmental specificity in the regulation of members of these auxin- and ethylene-responsive gene families by the DGT gene product.

RESULTS

The *dgt* Mutation Affects Fruit Size and Internal Anatomy of Tomato Fruit

We investigated the effects of the *dgt* mutation on fruit development using three different *dgt* alleles (*dgt* $^{1\text{-}1}$, *dgt* $^{1\text{-}2}$, and *dgt* ^{dp}) produced by three different mechanisms (spontaneous, ethyl methanesulfonate, and x-ray induced, respectively). The presence of the three different *dgt* alleles in four different isogenic and near-isogenic tomato varieties allowed us to evaluate possible allele- and background-specific effects. The *dgt* mutation affects the size, weight, and internal anatomy of tomato fruit. Fruit size is clearly reduced in *dgt* plants irrespective of genetic background (Fig. 1). Fruit weight, number of locules, and number of seeds per fruit varies for each genetic background, but with few exceptions, these characteristics are significantly reduced by the *dgt* mutation in each genetic background in both greenhouse and

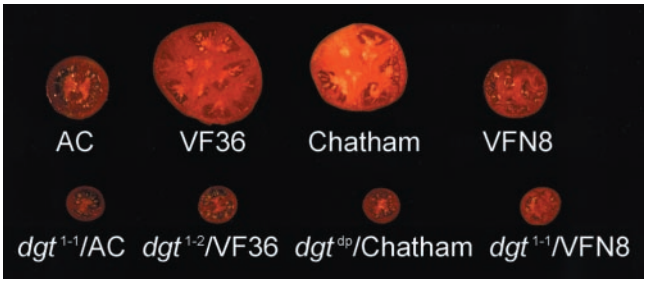


Figure 1. The *dgt* mutation affects fruits characteristics irrespective of genetic background. Top, Ripe wild-type tomato fruits (Alisa Craig [AC]). Bottom, Ripe *dgt* tomato fruits in corresponding genetic backgrounds. Fruits were obtained from the greenhouse experiment.

growth chamber experiments (Table I). The largest difference between *dgt* and wild-type fruits is in number of seeds per fruit, followed by fruit weight, and then by number of locules per fruit. The *dgt* mutation has less of an effect on all of these characteristics when plants are grown in growth chambers. The largest differences between greenhouse and growth chamber results are observed for the number of seeds per fruit, whereas the smallest differences are in fruit weight (Table I).

Relative growth rate is also significantly lower in *dgt* compared with wild-type fruits. For example, in a typical experiment comparing VFN8 and *dgt* $^{1\text{-}1}$/VFN8 fruits, the wild-type relative growth rate was 0.10 cm d^{-1}, whereas the *dgt* relative fruit growth rate was 0.04 cm d^{-1} ($P = 0.05$; data not shown). Fruit set is also dramatically decreased by the *dgt* mutation, with 70% to 93% reduction under greenhouse conditions and 11% to 64% reduction in plants grown in growth chambers (data not shown).

Mutation of the *Dgt* Gene Delays the Onset of Fruit Development

In addition to affecting fruit characteristics, the *dgt* lesion delays the onset of reproductive development, measured as the number of days from planting to anthesis. Depending on the allele and parent line tested, the *dgt* lesion delays first anthesis from 35 to 70 d in the greenhouse and from 23 to 49 d in growth chambers. Figure 2 shows a representative developmental time course for *dgt* $^{1\text{-}1}$/VFN8 and the wild-type isogenic parent line, VFN8. The number of internodes produced before flowering also increases in the *dgt* mutant. For example, *dgt* $^{1\text{-}1}$/VFN8 produced eight more internodes before flowering than did wild-type VFN8 plants in a typical greenhouse experiment (data not shown). Similar results were seen with the other *dgt* alleles and genetic backgrounds.

The time necessary for fruits to progress from anthesis (A) to breaker (B) stage, the first appearance of orange color at the blossom end of fruit, is also dramatically increased in all *dgt* mutant alleles under greenhouse conditions. For example, *dgt* $^{1\text{-}1}$/VFN8 requires 83 more d to develop from anthesis to B than wild-type VFN8 (Fig. 2). When grown in growth chambers, the time from anthesis (A) to breaker (B) is similar for wild-type and corresponding *dgt* fruits. The time from B to red ripe (R) is not changed significantly by the *dgt* lesion for any mutant allele background comparison in either condition tested (Fig. 2; data not shown).

Ethylene Evolution during *dgt* and Wild-Type Fruit Development

To test whether the *dgt* mutation affects ethylene production, the rate of ethylene evolution was measured in mutant and wild-type fruits at several stages of development. In all cases, ethylene production is low in preclimateric fruit and increases at the onset of ripening. A peak in ethylene production occurs at the orange (O) stage and declines slightly later. Although minor differences are observed at certain stages, no clear pattern of differential ethylene production between mutant and wild-type fruits is found in the four genetic backgrounds at any stage of fruit growth or ripening (Table II).

Table I. *The dgt mutation affects fruit size and internal anatomy of tomato fruit*

Effect of the *dgt* mutation on fruit weight and internal anatomy of tomato fruits. Mean fruit weight, locules, and seed nos. were measured at ripening in both greenhouse and growth chamber experiments from at least 30 fruits. Values within a column followed by the same letter are not significantly different at the $P = 0.05$ level by Tukey's Studentized Range (HSD, honest significant difference) test.

Genotype	Fruit Wt		No. of Locules per Fruit		No. Seeds per Fruit	
	Greenhouse	Growth chamber	Greenhouse	Growth chamber	Greenhouse	Growth chamber
	g					
AC	32.6 bc	47.5 c	2.1 cd	2.1 d	82.5 a	128 a
dgt $^{1\text{-}1}$/AC	6.2 d	23.0 de	1.1 e	2.0 d	13.2 cd	61.9 df
VFN8	38.2 b	69.8 b	4.9 b	5.6 b	70.5 ba	96.6 bc
dgt $^{1\text{-}1}$/VFN8	9.8 d	23.3 de	1.3 ed	3.5 c	14.6 cd	62.5 ef
VF36	64.9 a	99.3 a	7.3 a	7.1 a	48.6 bc	101.9 b
dgt $^{1\text{-}2}$/VF36	22.2 c	44.4 cd	3.1 cd	5.1 b	27.4 cd	82.4 bf
Chatham	28.8 c	29.3 ce	4.5 b	5.0 b	72.3 ba	93.1 bde
dgt ^{dp}/Chatham	8.4 d	20.7 e	2.7 cd	3.7 c	23.0 cd	75.2 cef

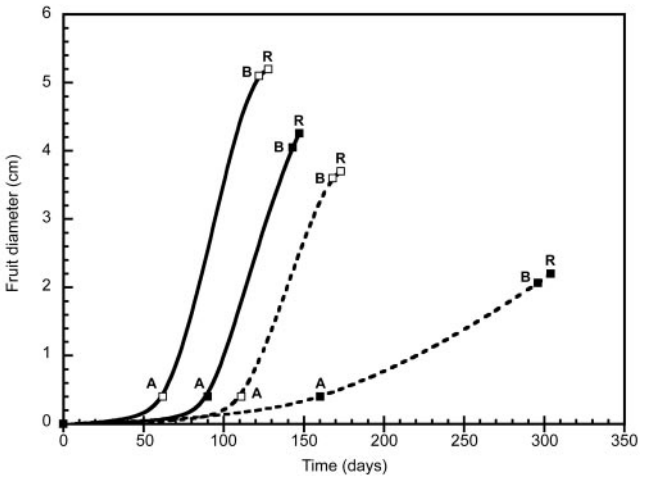


Figure 2. The *dgt* mutation delays the onset of reproductive development and reduces fruit size. Fruit diameters were measured three times per week from the time of fruit set until ripeness and increases were plotted against time from planting to ripeness. Values represent means of at least six fruits. Diameters at anthesis (A), breaker (B), and red ripe (R) are shown. —■—, VFN8, greenhouse; —□—, VFN8, growth chamber; --■--, *dgt* $^{1\text{-}1}$/VFN8, greenhouse; --□--, *dgt* $^{1\text{-}1}$/VFN8, growth chamber.

Differential Expression of ACS Genes during Fruit Development in *dgt* and Wild-Type Tomato Plants

To investigate whether the *dgt* mutation affects the expression of ethylene-responsive and nonresponsive genes from a single gene family during fruit development, relative RNA expression patterns were determined using real-time reverse transcriptase (RT)-PCR for each *LeACS* gene family member in *dgt* and wild-type tomato fruits. Transcript levels of *LeACS* genes were normalized to transcript levels of *RPL2* (ribosomal protein large subunit 2; Fleming et al., 1993) to allow quantification of gene expression relative to an endogenous control.

Among the eight members of the *LeACS* gene family, transcripts from *LeACS 2*, *4*, and *6* are detected in both wild-type and *dgt* fruits (Fig. 3, A–C). *LeACS7* mRNA is detected in wild-type fruits at 15 DPA and the immature green (IG) stage, but not in *dgt* fruits at any stage (Fig. 3D). Similarly, *LeACS7* transcripts are also present in wild-type hypocotyls but not detected in *dgt* hypocotyls (data not shown). *LeACS1B*, *3*, and *5* transcripts were detected at the IG stage in both wild-type and *dgt* fruits only when higher concentrations of template were used (0.25 versus 0.025 μg μL^{-1}; data not shown), indicating that transcripts from these genes occur at lower abundance than *LeACS2*, *4*, and *6* transcripts in tomato fruits. The *LeACS1A* gene was expressed in wild-type fruits at low relative levels in all developmental stages evaluated. In *dgt* fruits, transcripts of the *LeACS1A* gene were detected only at the mature green (MG) and O stages, where their relative levels were comparable with those found in the wild-type fruits (data not shown).

Expression of *LeACS2* and *4* is similar in *dgt* and wild-type fruits (Fig. 3, A and B) and follows the well-documented ripening-related patterns of expression (Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992; Lincoln et al., 1993). Transcripts of these genes are not detected in preclimateric stages of fruit development (15 DPA, IG, and MG), increase from the B to the O stage, and decline thereafter (Fig. 3, A and B). In contrast, the *LeACS6* gene is expressed at 15 DPA and the IG and MG stages in wild-type fruits, but is not detected during ripening (Fig. 3C). In *dgt* fruits, *LeACS6* is expressed at 15 DPA and IG stage, and the expression level at 15 DPA is 5-fold higher than in wild-type fruits (Fig. 3C). The *LeACS6* gene is expressed at lower relative levels than *LeACS2* and *4* in wild-type fruits (Fig. 3, A–C), whereas in *dgt*, the relative expression levels of *LeACS6* at 15 DPA are comparable with those of *LeACS4* in the O stage (Fig. 3, B and C).

Differential Expression of Members of the *LeIAA* Gene Family during Fruit Development in Wild-Type and *dgt* Tomato Plants

To determine how the expression of the *LeIAA* genes in fruits compares with patterns previously reported in seedlings (Nebenführ et al., 2000), we analyzed endogenous expression of *LeIAA* genes in wild-type and *dgt* fruits using RT-PCR. Although *LeIAA1*, *3*, *4*, *6*, and *8* are constitutively expressed in all five fruit developmental stages, *LeIAA2*, *10*, and *11* transcripts are only present at the IG stage of tomato development. *LeIAA5* is expressed only at the IG and O developmental stages (Fig. 4). No differences between *dgt* and wild-type expression were observed for any of the genes using this technique.

Further investigation of *LeIAA2*, *8*, *10*, and *11* using real-time quantitative RT-PCR revealed significant differences in the relative expression levels of *LeIAA2* and *8*. Transcripts from *LeIAA2*, *10*, and *11* are detected in both wild-type and *dgt* fruits only at the 15-DPA and IG stages (Fig. 5, A, C, and D). In contrast, *LeIAA8* is constitutively expressed in both wild-type and dgt fruits throughout all developmental stages evaluated (Fig. 5B). Although there are no significant differences in the relative expression levels of *LeIAA10* and *11* between *dgt* and wild-type fruits, *dgt* fruits contain considerably higher levels of *LeIAA2* transcript at the 15-DPA stage compared with wild-type fruits (Fig. 5, A, C, and D). Relative transcript levels of *LeIAA8* are higher in *dgt* than in wild-type fruits during several stages of fruit development, most notably at the early stages (Fig. 5B).

DISCUSSION

Although the auxin-resistant *dgt* mutant has been reported to exhibit normal reproductive behavior

Table II. *Ethylene evolution during dgt and wild-type fruit development*

Ethylene production from *dgt* and wild-type tomato fruits during development. Fruits were harvested at the following stages: immature green (IG), mature green (MG), breaker (B), orange (O), red ripe (R), and full ripe (FR). Values are expressed in nanoliters per gram per hour (±SE of the mean, n = 4–12 fruits). Fruits were obtained from the growth chamber experiment.

Genotype	Fruit Stages					
	IG	MG	B	O	R	FR
			nL g h^{-1}			
AC	0.11 ± 0.26	2.99 ± 1.78	3.44 ± 0.83	5.12 ± 1.70	4.63 ± 2.99	0.89 ± 0.53
dgt $^{1\text{-}1}$/AC	0	2.74 ± 0.42	7.56 ± 2.90	9.38 ± 4.27	2.03 ± 0.82	0.97 ± 0.29
VFN8	0	2.25 ± 0.62	5.21 ± 2.62	2.96 ± 1.75	4.09 ± 2.94	3.71 ± 2.46
dgt $^{1\text{-}1}$/VFN8	0.18 ± 0.21	1.32 ± 0.91	5.74 ± 2.49	11.06 ± 2.61	2.71 ± 1.47	5.90 ± 2.90
VF36	0	0.46 ± 0.09	3.68 ± 0.32	6.79 ± 2.40	6.11 ± 1.39	2.50 ± 1.70
dgt $^{1\text{-}2}$/VF36	0.04 ± 0.09	4.30 ± 2.10	4.24 ± 1.13	8.77 ± 2.58	5.90 ± 1.70	1.17 ± 0.11
Chatham	0	0.37 ± 0.17	9.64 ± 3.43	11.96 ± 0.55	3.24 ± 0.99	1.29 ± 0.31
dgt ^{dp}/Chatham	0.07 ± 0.18	1.60 ± 0.6	12.1 ± 3.6	12.22 ± 2.9	12.29 ± 4.76	6.00 ± 1.70

(Fujino et al., 1988a; Ludford, 1995), we found that the *dgt* lesion dramatically reduces fruit size (Fig. 1), fruit weight, number of locules, number of seeds (Table I), and fruit set (data not shown), irrespective of genetic background or mutant allele.

Final fruit size results from the number of cells within the ovary before fertilization, the number of seeds, the number of cell divisions that occur in the developing fruit after fertilization, and the extent of cell expansion (Gillaspy et al., 1993). Locule number is also positively correlated with final fruit weight in tomato (Houghtaling, 1935; Yeager, 1937; MacArthur and Butler, 1938; Lippman and Tanksley, 2001). The involvement of all these factors in determining final fruit size clearly indicates the complexity of this phenomenon. The reduction in fruit weight in *dgt*—be-

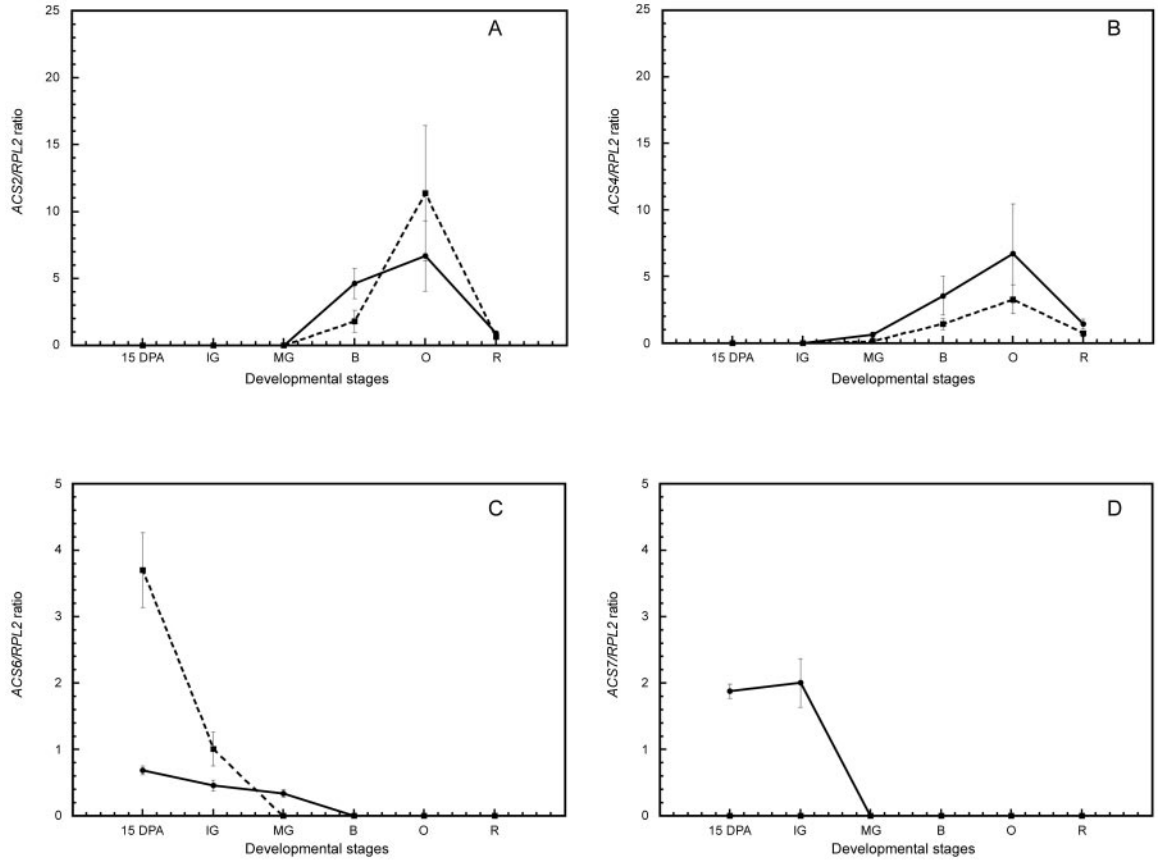
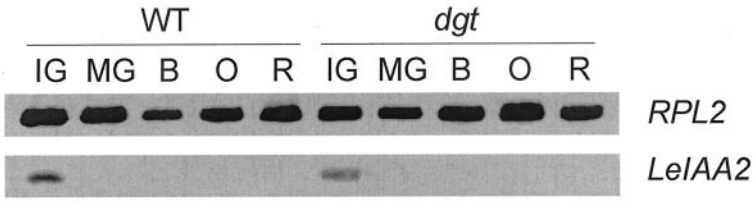


Figure 3. Differential expression of *LeACS* genes during fruit development in wild-type and *dgt* plants. Fruits of both *dgt* (dotted lines) and wild-type (solid lines) were harvested at the following stages: 15 DPA, IG, MG, B, O, and R. Expression levels of *LeACS* transcripts relative to *RPL2* were measured via real-time RT-PCR as described in "Materials and Methods." A, *LeACS2*. B, *LeACS4*. C, *LeACS6*. D, *LeACS7*. Note different scales.

A.



B.

	Fruit developmental stages					
	IG	MG	B	O	R	
WT	+	+	+	+	+	*LeIAA1*
dgt	+	+	+	+	+	
WT	+	−	−	−	−	*LeIAA2*
dgt	+	−	−	−	−	
WT	+	+	+	+	+	*LeIAA3*
dgt	+	+	+	+	+	
WT	+	+	+	+	+	*LeIAA4*
dgt	+	+	+	+	+	
WT	+	−	−	+	−	*LeIAA5*
dgt	+	−	−	+	−	
WT	+	+	+	+	+	*LeIAA6*
dgt	+	+	+	+	+	
WT	+	+	+	+	+	*LeIAA8*
dgt	+	+	+	+	+	
WT	+	−	−	−	−	*LeIAA10*
dgt	+	−	−	−	−	
WT	+	−	−	−	−	*LeIAA11*
dgt	+	−	−	−	−	

Figure 4. Differential expression of members of the *LeIAA* gene family during fruit development in wild-type and *dgt* plants. Fruits were harvested at the following stages: IG, MG, B, O, and R. A, Gene expression was analyzed by RT-PCR. Total RNA (2.5 μg) was used in the RT reaction in a final volume of 20 μL. The cDNAs generated were subsequently used in a 25-μL PCR reaction in the presence of specific primers for each *LeIAA* gene as well as the *RPL2* control. The RT-PCR products were separated on a 1.5% (w/v) agarose gel stained with ethidium bromide. A representative experiment is shown. B, Presence (+) or absence (−) of *LeIAA* transcripts at different stages of fruit development in wild-type and *dgt* tomatoes. Data was obtained as described for A. No cDNA clone has been isolated for *LeIAA7*. *LeIAA9* exhibited very low and erratic expression levels, precluding accurate analysis.

tween 29% and 81% depending on the allele, parent line, and growth conditions (Table I)—may be explained in part by the reduced number of seeds. Seed number is proposed to enhance fruit growth by controlling cell division in the surrounding tissue (Varga and Bruinsma, 1986).

Relative fruit growth rate, measured from the time of fruit set (roughly defined as the point at which the ovary diameter triples from that at anthesis) until the B stage, is significantly lower in *dgt* fruits. Because cell division in tomato ovaries reportedly occurs for only 7 to 10 d after fertilization (Mapelli et al., 1978; Varga and Bruinsma, 1986) and most cell expansion stops at the B stage, the measured period roughly corresponds to the cell expansion phase of fruit development. The lower relative fruit growth rate found in *dgt* fruits suggests that reduced cell expansion also plays a role in the smaller final size of *dgt* fruits. When the growth of wild-type and *dgt* developing fruits was followed by measuring the increase in ovary diameter at 5-d intervals from pre-anthesis to 20 DPA, the larger size of wild-type ovaries was evident by 5 DPA (data not shown). Taken together, our results suggest that both cell division and cell expansion are involved in the generation of the smaller fruit size in *dgt*.

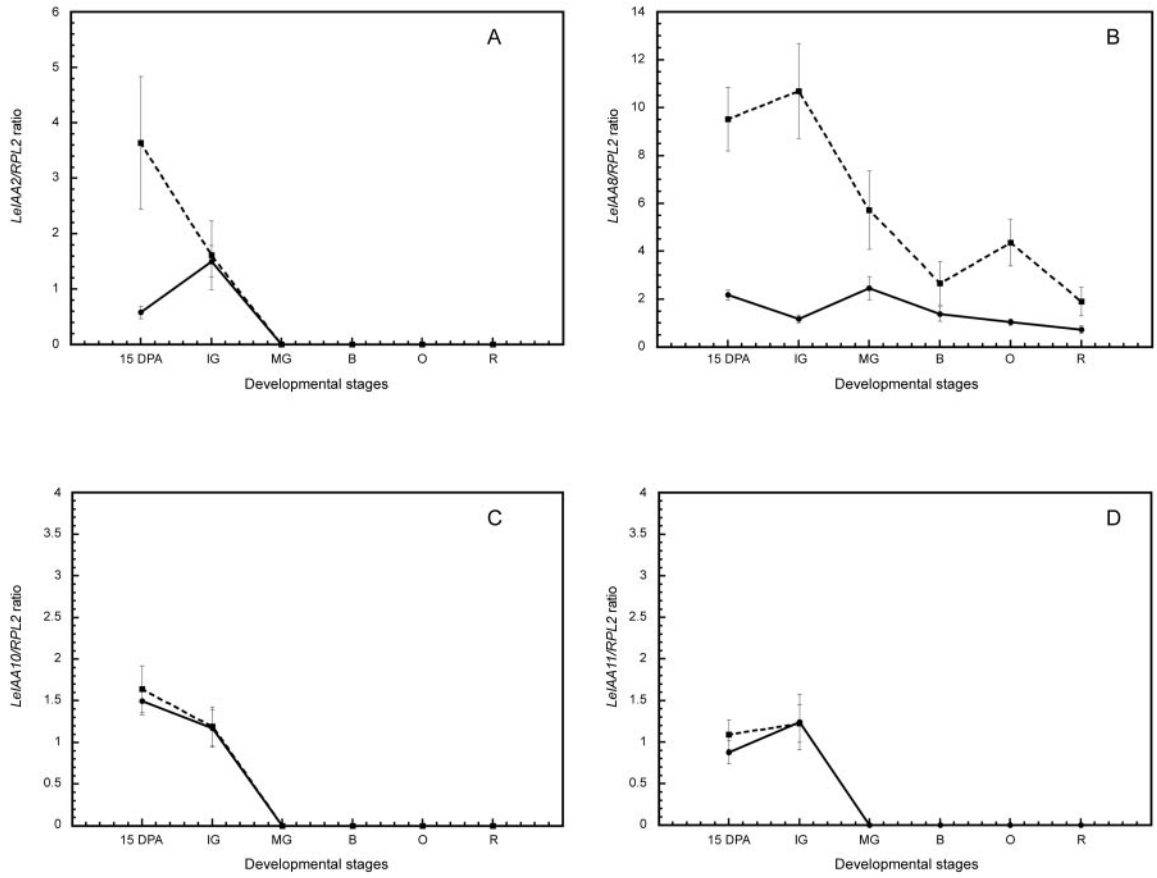


Figure 5. Differential expression of four members of the *LelAA* gene family during fruit development in wild-type and *dgt* plants. Fruits of both *dgt* (dotted lines) and wild type (solid lines) were harvested at the same stages as those in Figure 3. Expression levels of *LelAA* transcripts relative to *RPL2* were measured via real-time RT-PCR as described in "Materials and Methods." A, *LelAA2*. B, *LelAA8*. C, *LelAA10*. D, *LelAA11*. Note different scales.

Fruit set is also greatly reduced by the *dgt* mutation. Auxins are implicated as part of the signal transduction pathway that controls fruit set in tomato (Gillaspy et al., 1993). We did not perform comparative studies of the auxin responsiveness of *dgt* fruits; therefore, *dgt* effects on fruit set cannot be directly tied to auxin. Because flowers were not manually pollinated, the lower fruit set may also be a result of an effect of the *dgt* mutation on pollen release rather than on fruit set directly.

Time to flowering, measured by the number of internodes produced before the reproductive switch and by the number of days from planting to anthesis, was significantly longer in *dgt* than in wild-type plants (Fig. 2). Of the several Arabidopsis *Aux/IAA* mutants exhibiting reproductive phenotypes as part of their related but distinct pleiotropic phenotypes, only the *shy2-2* mutant, which exhibits early flowering, is reported to affect developmental time (Tian and Reed, 1999).

The *dgt* lesion also affects the developmental timing of the early stages of fruit growth. Although the time required for fruits to progress from anthesis to B is dramatically increased by the *dgt* mutation under greenhouse conditions, it is comparable with wild-type fruits under more controlled growth chamber conditions (Fig. 2). Taken together with the effects of growth conditions on fruit weight, number of locules, and seeds (Table I), these results indicate that reproductive development is more environmentally plastic in *dgt* plants. The average temperature varied between the greenhouse (28.2°C ± 3°C, days; and 15°C ± 2.3°C, nights; with a diurnal temperature range over the growing season between 23.3°C and 37.7°C) and the growth chambers (25°C ± 1.5°C, days; and 15°C ± 1.5°C, nights). However, differences in humidity, light levels, photoperiod, and/or CO_2 levels may also influence the increased environmental plasticity of reproductive development in *dgt*. Detailed measurements of water potential, photosynthesis, and leaf area were outside the scope of this study, but will be needed to more exactly identify the environmental conditions that influence the plasticity of the *dgt* reproductive phenotype.

It is possible that some effects of the *dgt* mutation on fruit set, seed number, fruit size, and developmental time are indirect results of the reduced leaf area, and root and vascular systems typical of *dgt* plants.

When reciprocal graftings between wild-type and *dgt* plants were performed, however, the mutant phenotype was maintained even in the presence of wild-type root stock (data not shown), indicating that the root biomass or structure is not responsible for the fruit differences. Photosynthetic rates are similar between *dgt* and wild-type plants (Lomax et al., 1993), but total leaf biomass may influence the final fruit characteristics.

Induction of ethylene synthesis via auxin has been reported (Yang and Hoffman, 1984; Yip et al., 1992). No clear pattern of differential behavior in total ethylene evolution between *dgt* and wild-type fruits was found at any stage of fruit development (Table II), which is consistent with the lack of significant change in developmental time from breaker to ripening in mutant versus wild-type fruits (Fig. 2). However, it remained possible that more subtle differences in isoform-specific ethylene evolution are involved in altered early fruit development in the *dgt* mutant.

Two systems have been proposed to explain the regulation of ethylene during plant development (for review, see Lelievre et al., 1997). System 1 provides for the basal level of ethylene present in vegetative tissues and preclimateric and non-climateric fruits, whereas system 2 is responsible for the high levels of ethylene production associated with ripening of climateric fruits and flower senescence (Oetiker and Yang, 1995). Specific members of the *LeACS* and *LeACO* gene families are proposed to regulate the transition from system 1 to 2 ethylene production (Nakatsuka et al., 1998; Barry et al., 2000). Based on their gene expression patterns, as well as their regulation by ethylene, *LeACS2* and *4* are proposed to mediate system 2 ethylene production, whereas *LeACS1A* and *6* function in system 1 in green fruit and vegetative tissue (Nakatsuka et al., 1998; Barry et al., 2000).

To investigate whether either system 1 or 2 is altered by the *dgt* mutation, we measured the expression levels of all known *LeACS* gene family members relative to *RPL2* from six developmental stages in both *dgt* and wild-type fruits using real-time RT-PCR (Fig. 3). The high sensitivity and specificity of real-time RT-PCR is important when analyzing the often low expression levels associated with the expression pattern of individual members of large gene families (Freeman et al., 1999). Our results generally agree with previous studies with respect to transcript occurrence; however, more subtle variations were observed via this more sensitive assay. Transcripts of *LeACS1B*, *3*, and *5* were only detected when higher concentrations of template were used, indicating that the abundance of these transcripts is low (data not shown). Previous studies that did not detect expression of *LeACS1B*, *LeACS5* (Nakatsuka et al., 1998; Barry et al., 2000), and *LeACS7* (Barry et al., 2000) in fruits used ribonuclease protection assays and northern-blot analysis, which are less sensitive and specific than real-time RT-PCR.

Expression of *LeACS2* and *4* was not detected in either wild-type or *dgt* fruits at the preclimateric stages, but increased equally in both genotypes with the onset of ripening (Fig. 3, A and B), indicating that system 2 is intact in mutant fruits and agreeing with our observations that ripening is unaffected by the *dgt* lesion. *LeACS6* is only expressed early in the development in both *dgt* and wild-type fruits, a pattern that has been linked to the regulation of system 1 ethylene synthesis in tomato fruit (Nakatsuka et al., 1998; Barry et al., 2000). Transcript levels of *LeACS6* were 4- to 5-fold higher in *dgt* versus wild-type fruits at 15 DPA (Fig. 3C). Expression of the *LeACS6* gene is subject to negative feedback regulation by ethylene (Nakatsuka et al., 1998; Barry et al., 2000) and it will be interesting to determine whether the intact DGT gene product plays a role in its regulation by ethylene or represses *LeACS6* expression via an alternate pathway. The greatly increased relative expression level of *LeACS6* in *dgt* at 15 DPA also suggests that alterations in early fruit development result, at least in part, from changes in system 1 ethylene production. The *LeACS1A* gene, which also participates in system 1 ethylene production, is expressed in wild-type fruits at all developmental stages evaluated. Very low relative levels of *LeACS1A* transcript found in *dgt* fruits precluded definitive conclusions regarding the effect of *dgt* on *LeACS1A* expression (data not shown).

The *LeACS7* gene, which has not previously been associated with system 1, is also exclusively expressed early in fruit development (15 DPA and IG). However, *LeACS7* transcripts were not detected in *dgt* fruits at any stage (Fig. 3D). *LeACS7* expression was also not detected in *dgt* hypocotyls but was present in wild-type hypocotyls (data not shown). *LeACS7* has been reported to play an early and transient role during flooding and wounding responses (Shiu et al., 1998); however, the significance of *LeACS7* in fruit development is not known. The accumulation of mRNAs from the auxin-inducible *LeACS3* and *5* genes requires a wild-type *Dgt* gene in hypocotyls (Coenen and Lomax, 2003). It may be that intact *Dgt* expression is also necessary for the expression of *LeACS7* and that the *LeACS7* gene product is also involved in system 1 regulation of early fruit development.

The significant changes in *LeACS* transcript accumulation in *dgt* fruit take place when ethylene production is not reliably detected. There could potentially be posttranscriptional regulation such that activity of the ACS protein is not directly related to the steady-state levels of *ACS* mRNA. Evidence does exist for posttranslational regulation of ACS (Woeste et al., 1999; Vogel et al., 1998). Alternatively, the activity of ACO may be insufficient to allow significant ethylene production at that point in develop-

ment. However, the correlation with the altered *dgt* fruit phenotype makes it most likely that the normal activity of LeACS1A, 6 and 7 during early fruit ontology is sufficient to modulate normal development, but too subtle to produce significant ethylene to be measured via gas chromatography (note the difference in scale between Fig. 3, C and D, and A and B).

Because *dgt* mutants are not affected in overall auxin metabolism or transport and auxin responsiveness is not completely abolished (Muday et al., 1995; Rice and Lomax, 2000), it has been proposed that the *dgt* lesion disrupts a specific step during early auxin signal transduction (Nebenführ et al., 2000). In hypocotyls, the *dgt* lesion specifically disrupts expression of a subset of *Aux/IAA* gene family members (*LeIAA5*, *8*, *10*, and *11*) while not affecting others (e.g. *LeIAA1–3*). The developmental specificity of *LeIAA* gene expression in fruits differs from that previously found in seedlings (Figs. 4–6). For example, although *LeIAA2* and *LeIAA10* are constitutively expressed in seedlings (Nebenführ et al., 2000), both genes are only expressed at the IG stage in fruits (Fig. 4). This finding suggests that specific LeIAA family members play different functional roles during fruit development versus seedling growth. Interestingly, all *LeIAA* genes measured are expressed at the IG stage of fruit development (Fig. 4). This result may indicate that participation of all *LeIAA* gene members is required during the IG stage of tomato fruit development when cell expansion is the primary process driving fruit growth (Gillaspy et al., 1993).

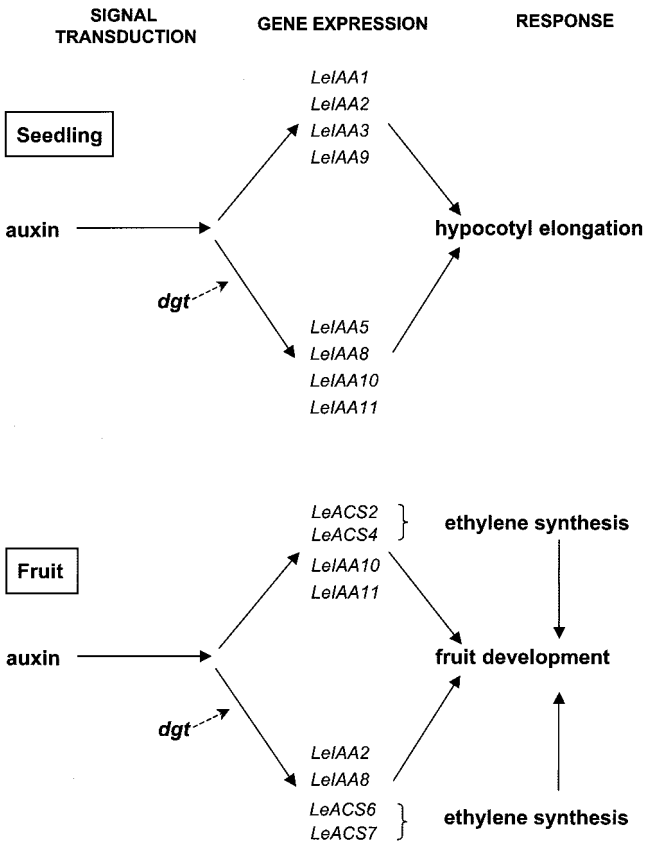


Figure 6. Diagram of gene expression of members of the *LeIAA* and *LeACS* gene families in tomato seedlings and fruits. Note the differential developmental specificity in the expression of members of the *LeIAA* gene family during hypocotyl elongation and fruit development in wild-type and *dgt* plants. The broken arrows labeled *dgt* indicate that the expression of the genes in that pathway is either enhanced or lowered by the *dgt* lesion.

Real-time RT-PCR was used to more precisely measure relative expression levels of a subset of *LeIAA* genes that differ both in their endogenous expression patterns during fruit development (Fig. 4) and in the effects of the DGT gene product on their auxin regulation in seedlings (Nebenführ et al., 2000). Both *LeIAA2* and *LeIAA8* are expressed at higher levels in *dgt* at early stages of fruit development compared with wild-type fruits (Fig. 5, A and B). In contrast, expression of *LeIAA10* and *11* is similar in wild-type and *dgt* fruits (Fig. 5, C and D). This is the opposite result from that found in hypocotyls, where the *dgt* mutation reduces transcript levels of *LeIAA8*, *10*, and *11* but has no effect on *LeIAA2* (Nebenführ et al., 2000). Thus, in different tissues and/or during distinct developmental processes, the DGT gene product regulates different members of the *LeIAA* gene family. The differences in expression between fruits (this study) and seedlings (Nebenführ et al., 2000) indicate either different functional roles for the *Aux/IAA* genes in regulating fruit and seedling development or different roles of DGT in these processes (Fig. 6). The *dgt* mutation does not seem to affect developmental specificity of *LeIAA* genes in terms of transcript occurrence but rather in terms of relative levels of expression.

Aux/IAA-mediated negative feedback has been proposed to allow tight regulation between auxin abundance and target gene expression in different cells (Reed, 2001), thus mediating tissue-specific responses to auxin during plant development. The *dgt* lesion may disrupt the function of *Aux/IAA* genes early in signal transduction and alter their role as tissue-specific mediators during the regulation of various developmental processes. This hypothesis could explain the highly pleiotropic phenotype of the *dgt* mutant. In this context, the up-regulation of *LeIAA2* and *8* in *dgt* fruits indicates that the intact *Dgt*-gene product functions as a negative regulator in wild-type fruit tissues (Fig. 5, A and B). Although expression of *LeIAA10* and *11* requires a functional DGT protein in hypocotyls, they appear to be regulated by a DGT-independent pathway during fruit development (Fig. 5, C and D).

To our knowledge, this study provides the first analysis of *Aux/IAA* gene expression in fruits, as well as the first comparison of gene expression patterns between the *LeIAA* and *LeACS* gene families during fruit development. The altered expression of specific members of the *LeIAA* and *LeACS* gene families in the *dgt* mutant suggests a role for those genes not only in

Table III. *Sets of real-time RT-PCR primers used to amplify gene-specific regions*

Gene	Primer Sequence (5′ → 3′)	Gene	Primer Sequence (5′ → 3′)
LeACS1A		*LeACS7*	
Sense	AGTATGCGATGAAATCTATGCTGCTA	Sense	TCTGGCACTGTTTTTAACTCACCTAA
Antisense	TCTGAATCCTGGAAATCCCAAG	Antisense	GGCACCAACTCGAAATCCTG
LeACS1B		*LelAA2*	
Sense	TTCTTGACAAGGACACGCTACG	Sense	AAGCGAGCTATGTTAAAGTGAGCA
Antisense	ATTCAATCATCTCCTCAACCATTTC	Antisense	CCGTTGTATCCATCTGTTTCTGAA
LeACS2		*LelAA8*	
Sense	CTACGCAGCCACTGTCTTTGAC	Sense	CAAATACGTGAAGGTAGCAGTTGAC
Antisense	TGATTCCGACTCTAAATCCTGGTAA	Antisense	ACACCATTTGTAAGGTCCATAAGCT
LeACS3		*LelAA10*	
Sense	CCAGGCCTCGTTAGTGTCATG	Sense	GACTTCTCAAAAGCTTGATCGAGAG
Antisense	ATCTCATCGTTGGAATAGATTGCA	Antisense	TGAAATCTTTCATTCCTTGGACAA
LeACS4		*LelAA11*	
Sense	TTGCGACGAAATATATGCTGCT	Sense	AAAGAACAGTTTTAACGGACGTGAA
Antisense	CACTCGAAATCCTGGAAAACCT	Antisense	GACTTATCTGCATCCTCCAATGCT
LeACS5		*RPL2*	
Sense	CACAGTATTCGATTGGCCAAAAT	Sense	CAGCGGATGTCGTGCTATGAT
Antisense	AAATCATGCCAACTCTGAAACCTG	Antisense	GGGATGCTCCACTGGATTCA
LeACS6			
Sense	TATGCAGCAACCGCGTTTAGT		
Antisense	TGTACGAGTAAATAATCCCAACCCTAA		

generating the *dgt* reproductive phenotype, possibly as downstream targets of DGT, but also in the early development of wild-type fruits.

The correlation between altered expression of specific *LeIAA* and *LeACS* gene family members and differences in *dgt* and wild-type reproductive development indicates that auxin responsiveness and ethylene biosynthesis play significant roles in early fruit development and demonstrates the importance of the early stages of ovary/fruit development as determinants of mature fruit characteristics in tomato. Further studies should elucidate the complex mechanisms that regulate final fruit size and morphology in tomato. It will be especially interesting to determine whether *dgt* reproductive characteristics are determined pre- or postanthesis, as well as the relative importance of cell number and size in determining final fruit size.

MATERIALS AND METHODS

Plant Material

Four varieties of wild-type tomato (*Lycopersicon esculentum* Mill.)—Alisa Craig (AC), Chatham, VFN8, and VF36—as well as three alleles of the *dgt* mutation were tested under greenhouse and growth chamber conditions in facilities at Oregon State University. The mutant alleles used in this study were *dgt* $^{1\text{-}1}$, a spontaneous mutation in VFN8 (the isogenic parent) that was also extensively backcrossed into AC (a near-isogenic parent); *dgt* $^{1\text{-}2}$, an ethyl methanesulfonate-induced mutation in VF36 (the isogenic parent); and *dgt* ^{dp} (formerly called *droopy*; Jones and Jones, 1996), an x-ray-induced mutation in Chatham (the isogenic parent).

Growth Conditions and Phenotypic Measurements

For the greenhouse experiment, 10 plants of each mutant and corresponding parent were transplanted 2 weeks after germination and grown under greenhouse conditions with supplemental lighting (14 h of light and 10 h of dark). Greenhouse air temperature was set at 25°C during the day and 16°C at night. For the growth chamber experiment, four plants of each line were transferred from the greenhouse to growth chambers 4 weeks after germination. One plant of each mutant and its corresponding parent were placed in each of four identical growth chambers. An additional mutant/parent pair was assigned to each chamber to test for chamber-variety interactions. Light was supplied by a 1,000-W metal halide lamp in each chamber; photon flux densities averaged 400 μmol m^{-2} s^{-1}. Plants in the growth chambers were subjected to the same day length and fertilizing conditions as used in the greenhouse experiment. Air temperatures were set at 25°C during the day and 15°C at night. Plants were watered twice daily by drip irrigation.

Individual flowers were tagged on the day of anthesis (flower opening) and dates from anthesis to B and ripening were recorded for each tagged flower. In addition, the dates for anthesis of the first flower on each plant, B, and ripening were recorded for at least five fruits from each plant for all varieties. Ripe fruits were individually analyzed with respect to fruit weight, number of locules, and number of seeds. The total number of flowers and fruits per plant were recorded at biweekly intervals to identify peak flowering times and to calculate percent fruit set (no. of fruits/no. of flowers). In the greenhouse experiment, fruit diameters were measured three times per week (about six fruits per plant) from the time of fruit set (roughly defined as the point at which the ovary triples in diameter compared with the diameter at anthesis) until the B stage to allow calculation of relative growth rate (diameter at B − diameter at fruit set/no. of days from fruit set to B). Finally, the number of internodes to first flower was recorded for each plant in both experiments.

Ethylene Evolution Measurements

Ethylene evolution in *dgt* and wild-type fruits was measured using a gas chromatograph (model GC-14A, Shimadzu, Kyoto) equipped with a flame ionization detector and a Poropak Q column (Waters, Milford, MA). Measurements were taken from growth chamber-grown fruits harvested at the following stages: IG (about 2–3 weeks after flowering), MG (pale-green color in fruit surface), B, O, R (red color and firm texture), and FR (red color and soft texture). At least four fruits from each variety and developmental stage were used. Fruits were harvested and maintained in open 135-mL containers for 1 h to reduce the effect of wound ethylene production caused by harvesting. The containers were then sealed with airtight serum stoppers (Fisher Scientific, Pittsburgh) and allowed to equilibrate for 1 h. A 1-mL headspace sample was withdrawn from the airtight container using a 1-mL gas-tight syringe (Hamilton Co., Reno, NV) and injected into the gas chromatograph.

RNA Extraction, Reverse Transcription, and RT-PCR

Total RNA was extracted from the pericarp of fruits at six developmental stages–15 DPA, IG, MG, B, O, and R—using a hot phenol method (Verwoerd et al., 1989). After extraction, RNA samples were treated with DNaseI (RQ1, Promega, Madison, WI). Complementary DNA was synthesized from 2.5 μg of total RNA using oligo(dT) primers and modified Moloney murine leukemia virus RT (SuperScript II; Life Technologies/Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Expression of the *LeIAA* gene family was analyzed by RT-PCR in the presence of specific primers for each *LeIAA* gene, as well as the *RPL2* control essentially as described by Nebenführ and Lomax (1998). Expression analysis of *LeIAA* gene family members was performed in VFN8 and *dgt* $^{1\text{-}1}$/VFN8 and repeated in Chatham and *dgt* ^{dp} with similar results.

Analysis of Gene Expression by Real-Time RT-PCR

Real-time RT-PCR was performed with an ABI Prism 7700 sequence detection system (Central Services Laboratory, Oregon State University) using the SYBR Green PCR master mix kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. All real-time RT-PCR reactions were performed in Chatham and *dgt* ^{dp} fruits. Transcript levels of *LeACS* and *LeIAA* genes in the RNA samples were normalized with transcript levels of *RPL2* to allow quantification of gene expression relative to an endogenous control. Primers for specific amplification of each cDNA were designed using the Primer Express software (Applied Biosystems), taking into account criteria such as product length, optimal PCR annealing temperature, and likelihood of primer self-annealing (Table III).

PCR reactions were performed in triplicate in a 25-μL volume using 500 nM each forward and reverse primers, 12.5 μL of SYBR green master mix, 5 μL of a 1:10 (v/v) dilution of cDNA: 25 μL water. Reactions were performed in MicroAmp 96-well plates (Applied Biosystems) covered with optical adhesive covers (Applied Biosystems). Samples were subjected to a two-temperature thermal cycling consisting of denaturation at 95°C for 15 s, followed by anneal extension at 60°C for 1 min. To distinguish specific product from nonspecific products and primer dimers, a melting curve was obtained immediately after amplification by using the ABI PRISM Dissociation Analysis software (Applied Biosystems). The melting curve results were verified by subjecting PCR products to agarose gel electrophoresis and identifying the bands by DNA sequence analysis (Central Services Laboratory, Oregon State University).

Statistical Analysis

All statistical analyses were performed using SAS software (SAS Institute, Cary, NC). Data were subjected to one-way ANOVA for comparison of means. The statistical significance of differences between means was determined using Tukey's Studentized Range (HSD, honest significant difference) test.

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