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# The *diageotropica* mutation alters auxin induction of a subset of the *Aux/IAA* gene family in tomato

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## Abstract

The *diageotropica* (*dgt*) mutation has been proposed to affect either auxin perception or responsiveness in tomato plants. It has previously been demonstrated that the expression of one member of the *Aux/IAA* family of auxin-regulated genes is reduced in *dgt* plants. Here, we report the cloning of ten new members of the tomato *Aux/IAA* family by PCR amplification based on conserved protein domains. All of the gene family members except one (*LeIAA7*) are expressed in etiolated tomato seedlings, although they demonstrate tissue specificity (e.g. increased expression in hypocotyls vs. roots) within the seedling. The wild-type auxin-response characteristics of the expression of these tomato *LeIAA* genes are similar to those previously described for *Aux/IAA* family members in *Arabidopsis*. In *dgt* seedlings, auxin stimulation of gene expression was reduced in only a subset of *LeIAA* genes (*LeIAA5*, *8*, *10*, and *11*), with the greatest reduction associated with those genes with the strongest wild-type response to auxin. The remaining *LeIAA* genes tested exhibited essentially the same induction levels in response to the hormone in both *dgt* and wild-type hypocotyls. These results confirm that *dgt* plants can perceive auxin and suggest that a specific step in early auxin signal transduction is disrupted by the *dgt* mutation.

*Abbreviations:* ARF, auxin response factor; *dgt*, *diageotropica*; IAA, indole-3-acetic acid; *LeIAA*, *Lycopersicon esculentum Aux/IAA*; MTRP, multiplex-titration RT-PCR; RPL2, ribosomal protein L2; SAUR, small auxin up-regulated RNA

## Introduction

The *diageotropica* (*dgt*) mutant of tomato (*Lycopersicon esculentum* Mill.) is characterized by a wide range of developmental and physiological defects, including a reduced gravitropic response, shortened internodes, lack of lateral roots, reduced vasculature, hyponastic leaves, increased anthocyanin and chlorophyll synthesis, and reduced apical dominance (Lomax *et al*., 1993; Zobel, 1974). Seedlings homozygous for the *dgt* mutation exhibit reduced sensitivity to the plant hormone auxin (indole-3-acetic acid, IAA) as demonstrated by the lack of auxin-induced elongation and ethylene production in hypocotyls (Jackson, 1979; Kelly and Bradford, 1986), and reduced auxin inhibition of root growth (Muday *et al*., 1995). Shoot apices of seedlings homozygous for the *dgt* mutation contain normal levels of IAA (Fujino *et al*., 1988), the rate of polar auxin transport in mutant hypocotyls is essentially normal (Daniel *et al*., 1989), and *dgt* roots display no alterations in several transport-related phenomena (Muday *et al*., 1995). Taken together, these physiological results indicate that the *dgt* mutation of tomato is likely to affect a specific step in auxin perception or signaling. For this reason, *dgt* provides an important tool for investigation of the molecular mechanisms underlying auxin-mediated processes.

The induction of several auxin-regulated genes by exogenously applied IAA is abolished in *dgt* seedlings, including a *SAUR* (small auxin up-regulated

RNA) gene and *LeAux*, a homologue of the *Aux/IAA* gene family (Zurek *et al*., 1994; Mito and Bennett, 1995). However, the auxin-regulated expression of another tomato gene, *Lepar*, was not found to be affected in the mutant (Mito and Bennett, 1995). This raises the possibility that the $dgt^+$ gene product regulates only one of multiple auxin response pathways. To further investigate this issue, we have chosen to analyze the effect of the *dgt* lesion on auxin regulation within a large gene family. The *Aux/IAA* gene family is represented by at least 25 members in *Arabidopsis* (Kim *et al*., 1997) and is characterized by the presence of four conserved domains (Conner *et al*., 1990). Most of the predicted Aux/IAA proteins contain two functional nuclear localization signals, a $\beta\alpha\alpha$ motif suggestive of DNA binding, and domains with the potential to form protein-protein interactions (Abel *et al*., 1994; Abel and Theologis, 1995). The Aux/IAA proteins have been shown to form homo- and heterodimers, and also to interact with the related group of ARF1 (auxin response factor 1)-like transcription factors (Kim *et al*., 1997; Ulmasov *et al*., 1997a). These characteristics together with rapid induction of the mRNA and short half-life of the proteins make the Aux/IAA gene products excellent candidates for signaling intermediates in auxin responses (Abel and Theologis, 1996). Indeed, overexpression of individual *Aux/IAA* genes in carrot protoplasts results in reduced auxin-induced expression from an auxin-responsive promoter (Ulmasov *et al*., 1997b) and semi-dominant, gain-of-function mutations in the *AtIAA3* and *AtIAA17* genes of *Arabidopsis* result in the auxin-resistant phenotypes exhibited by *shy2* (Tian and Reed, 1999) and *axr3* (Rouse *et al*., 1998) mutants.

In *Arabidopsis*, individual members of the *Aux/IAA* family exhibit varying kinetics with respect to gene expression in response to auxin, which has been interpreted as a complex regulatory network with several hierarchical levels (Abel *et al*., 1995). We reasoned that the homologous *Aux/IAA* genes in tomato would be suitable for testing whether the *diageotropica* mutation affects a specific step in early auxin signaling or has a more general effect. In this study, we report the isolation and characterization of partial clones representing eleven members of the *Aux/IAA* gene family in tomato (*LeIAA1–11*). Expression studies in *dgt* seedlings demonstrate that only a subset of the *LeIAA* genes are affected by the lesion and suggest that the $dgt^+$ gene is involved in an early step in the regulation of gene expression by auxin.

## Materials and methods

### *Plant material and hormone treatments*

Seeds of tomato (*Lycopersicon esculentum* Mill.) cv. VFN8 and the isogenic, single-gene mutant *dgt* were surface-sterilized for 10 min in 20% household bleach, sown onto moist filter paper (Whatman 3MM paper, Maidstone, UK) and grown in constant darkness at 28 °C. The hypocotyls of 5-day old etiolated seedlings were harvested and cut into 5 to 10 mm sections. Endogenous auxin was depleted from the sections by pre-incubation in the dark in a 1% sucrose, 10 mM MES buffer (pH 6.0) for 2 h, after which the sections were transferred to fresh buffer with the indicated concentrations of indole-3-acetic acid (IAA). IAA was added as an ethanolic solution; the ethanol concentration was held constant at 0.1% during all hormone and control treatments. After incubation in the dark for the indicated times, the hypocotyl sections were briefly blotted onto filter paper and frozen in liquid nitrogen.

### *RNA extraction*

RNA extraction followed a published procedure (Chomczynski and Sacchi, 1987). Briefly, frozen hypocotyl sections (or the various tissues described in Figure 6) were ground to a fine powder in a mortar. Extraction buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl, 0.7% 2-mercaptoethanol) was added at 1.5 ml per gram fresh weight (FW) and the slurry allowed to thaw at room temperature. After addition of 150 $\mu$l/g FW 2 M sodium acetate, a phenol extraction was performed (1.5 ml/g FW water-saturated phenol and 300 $\mu$l/g FW chloroform/isoamyl alcohol, 49:1). The resulting aqueous phase was re-extracted with an equal volume of chloroform/isoamyl alcohol (49:1), followed by isopropanol precipitation, resuspension of the pellet, and LiCl precipitation. The resulting RNA pellet was resuspended in RNase-free water and precipitated with ethanol and 23 mM NaCl. This final pellet was resuspended in RNase-free water at ca. 50 $\mu$l/g FW. RNA concentrations were determined spectrophotometrically.

### *PCR with degenerate primers and cloning of products*

Genomic templates were isolated according to Rogers and Bendich (1994). cDNA templates were generated by reverse transcription with SuperScript II (Life

Technologies, Gaithersburg, MD) primed with oligo-(dT) according to manufacturer's instructions, using total RNA from auxin-treated hypocotyl segments from etiolated seedlings harvested as described above.

PCR amplification of *LeIAA* genes from tomato genomic DNA or cDNA was performed with degenerate primers targeting conserved domains II and IV of the *Aux/IAA* gene family (Abel *et al.*, 1995). The primers B-DD2 (5′-ATGGATCCGTNGTNGGNTGGCCNCC) and R-DD4 (5′-GCGAATTCATCCARTCNCCRTCYTTRTC) were used at a concentration of 300 nM each in a 25 $\mu$l reaction. 300 ng of genomic DNA or 1 $\mu$l of cDNA (1/20 of a reverse transcription reaction derived from 5 $\mu$g of total RNA from hypocotyl segments treated with auxin as described above) were used as template in an amplification reaction of 35 cycles (94 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s) with 1 unit of *Taq* DNA polymerase (Life Technologies). Since the primers were designed with *Bam*HI and *Eco*RI restriction sites near the ends (underlined), the amplification products were double-digested with both enzymes and cloned into a similarly cut vector (pGEM4Z, Promega, Madison, WI) by standard molecular biology protocols (Sambrook *et al.*, 1988). Colonies were screened by PCR with the same degenerate primers and positive clones were classified according to insert size.

*Sequence analysis*

Nucleic acid sequences of the clones were determined according to manufacturer's instructions with either a Sequenase Kit (US Biochemicals, Cleveland, OH) or an ABI Prism 373A Automated DNA Sequencer (Perkin Elmer, Seattle, WA). At least two independent clones were sequenced for most of the genes. Exceptions were *LeIAA1*, *7*, and *9* where only one clone each was obtained. Alignments of protein sequences deduced from the tomato *LeIAA* genes and from the nucleic acid sequences for other *Aux/IAA* and *ARF* genes from genetic databases were done with ClustalW (Thompson *et al.*, 1994) and corrected manually. Phylogenetic analysis based on this alignment was carried out with the Neighbor program of the PHYLIP v3.5 package (Felsenstein, 1989; available at http://evolution.genetics.washington.edu/phylip.html). Bootstrap values were calculated with the Consense program (PHYLIP) based on 100 bootstrapped sequences.

*RNase protection assays*

RNase protection assays were carried out essentially according to manufacturer's instructions using either an RPA III Kit (Figure 4; Ambion, Austin, TX) with $^{33}$P-labeled probes or an RPA II Kit with modifications. Modifications to the original RPA II protocol included: (1) the $^{35}$S-labeled probe was gel-purified (150 000 to 200 000 cpm per assay); (2) hybridization of probe and sample followed the 'streamlined' protocol; (3) precipitation was achieved by adding 200 $\mu$l Solution Dx and 200 $\mu$l ethanol; and (4) gels were rinsed in distilled water for 20 min before drying. Films were exposed for 7 to 21 days, depending on the amount of RNA in each assay. Developed films were scanned into a computer (Personal Densitometer, Molecular Dynamics) at a resolution of 100 $\mu$m. Band intensities were determined with the Gel Plotting Macros in NIH Image (available at http://rsb.info.nih.gov/nih-image/Default.html). *LeIAA* band intensities were normalized to the band intensity of ribosomal protein L2 (*RPL2*; Fleming *et al.*, 1993), which was included in all hybridization reactions as a non-auxin-responsive control gene.

*Expression analysis by multiplex-titration RT-PCR*

Relative expression levels of *LeIAA* genes were determined by multiplex-titration RT-PCR (MTRP) as described (Nebenführ and Lomax, 1998). Briefly, cDNA derived by reverse transcription from total RNA was serially diluted in four-fold steps and each individual dilution step was used as template in a PCR reaction. Gene-specific primers targeting three different *LeIAA* genes as well as a control gene (*RPL2*) were included in each reaction. The dilution step at which the template concentration became limiting was used as a measure of the relative abundance of transcript in the original RNA preparation.

## Results

*Cloning, sequencing and phylogenetic analysis*

Members of the *Aux/IAA* family of auxin-regulated genes are characterized by four conserved domains (I through IV) which are separated by intervening regions of variable length and sequence (Conner *et al.*, 1990). Degenerate PCR primers based on domains II and IV (Abel *et al.*, 1995) were used to amplify a

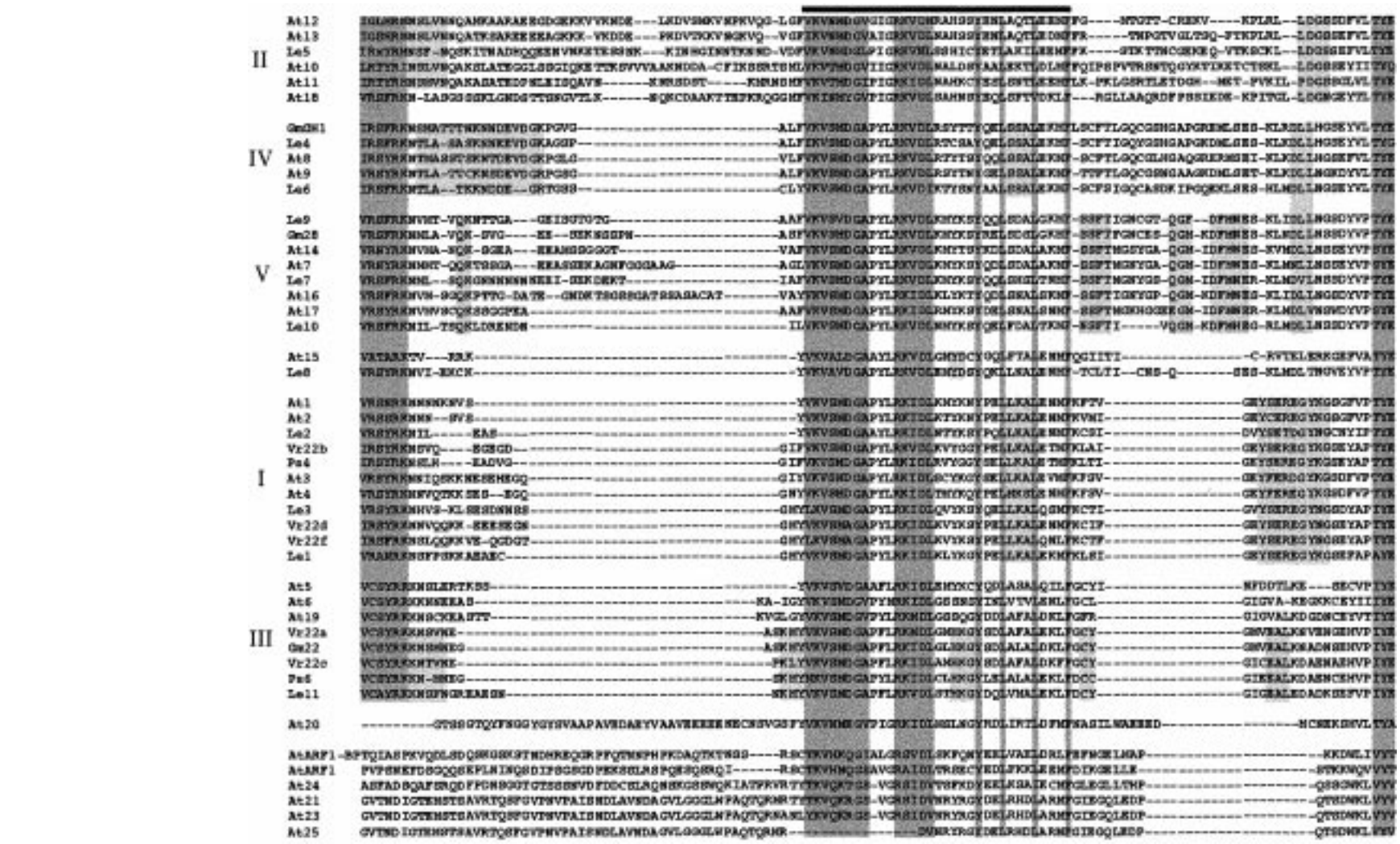


*Figure 1.* Alignment of LeIAA and published Aux/IAA peptide sequences. Deduced amino acid sequences between conserved domains II and IV are grouped to reflect the phylogenetic relationships depicted in Figure 2. The thick line above the alignment indicates the $\beta\alpha\alpha$ motif (conserved domain III). Residues conserved in most genes are marked in dark gray. Sequence motifs that are characteristic of individual subfamilies are highlighted in light gray. Names of *Aux/IAA* genes are abbreviated to include only the species designator and the gene number (e.g. Le1 = *LeIAA1*). The *AtARF* genes and *AtIAA21* to *25* are related but not members of the *Aux/IAA* family (Kim *et al*., 1997; Ulmasov *et al*., 1999) and have been included as an outgroup. Roman numerals designate the individual subfamilies (see text). Le, *Lycopersicon esculentum* (this study and Mito and Bennett, 1995); At, *Arabidopsis thaliana* (Conner *et al*., 1990; Abel *et al*., 1995; Kim et al., 1997); Gm, *Glycine max* (Ainley *et al*., 1988; Ulmasov *et al*., 1997b); Ps, *Pisum sativum* (Oeller *et al*., 1993); Vr: *Vigna radiata* (Yamamoto *et al*., 1992; Hashimoto and Yamamoto, 1997).

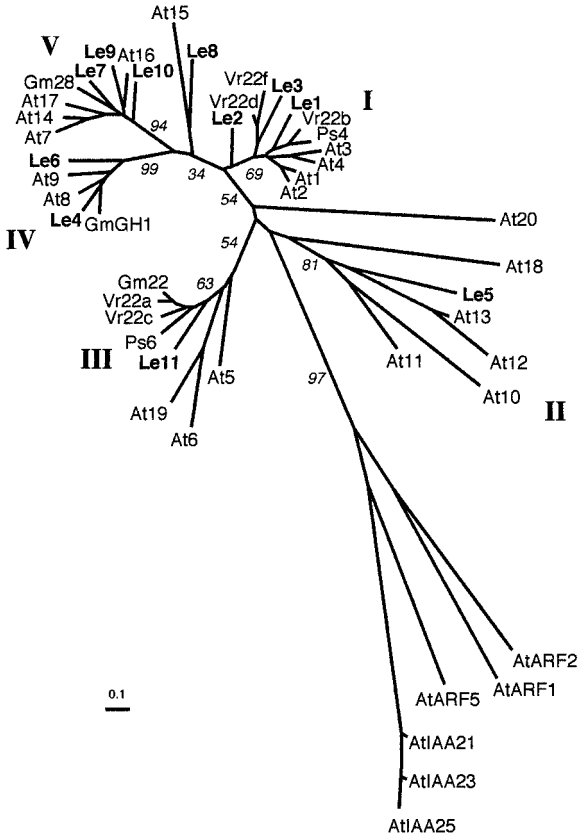


*Figure 2.* Phylogenetic analysis of *LeIAA* and related genes. Unrooted phylogenetic tree calculated with the Neighbor-Joining method (Saitou and Nei, 1987) based on amino acid alignment of the region depicted in Figure 1. Branch lengths represent the calculated distances between the sequences. Italicized numbers are bootstrap values from a consensus tree of similar topology. Roman numerals designate individual subfamilies (see text). For sequence names and sources, see Figure 1.

number of fragments from both genomic and cDNA templates derived from tomato seedlings. Cloning of the amplification products yielded clones with eleven different sequences named *LeIAA1* through *11* (Table 1). *LeIAA1* corresponds to *LeAux*, which was previously isolated by Mito and Bennett (1995). The presence of conserved domain III in all of the cloned sequences (Figure 1) confirmed that the *LeIAA* genes are members of the *Aux/IAA* family. *LeIAA2* through *LeIAA11* each contain at least one intron between domains II and IV based on the difference in size of PCR products generated using gene-specific primers with cDNA and genomic templates (Table 1 and data not shown). Two different cDNA clones representing different splice forms were isolated for *LeIAA2*.

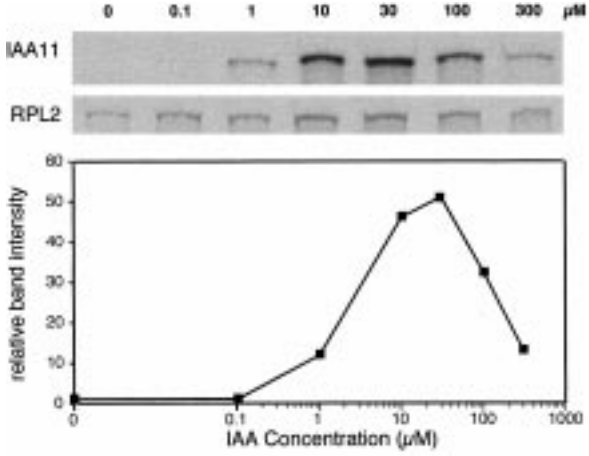


*Figure 3.* Concentration dependence of auxin induction of *LeIAA11*. 15 $\mu$g total RNA from hypocotyl segments of 5-day old etiolated wild-type tomato seedlings treated for 2 h with the indicated concentrations of indole-3-acetic acid were incubated with a radiolabeled antisense probe for gene *LeIAA11*. Fragments protected from degradation by RNase were separated on a polyacrylamide gel and visualized by autoradiography. Lane numbers correspond to IAA concentration ($\mu$M). Relative band intensities were determined densitometrically and normalized to the control gene (*RPL2*).

Phylogenetic analysis of the *LeIAA* genes and all published sequences of the *Aux/IAA* family was conducted to assess the relation of the tomato genes to those from other species and to determine their distribution within the family. The sequences included in this analysis included both typical *Aux/IAA* genes which contain all four conserved domains, as well as those in which the region of the protein amino-terminal to domain III is replaced with other sequences (AtARF1-related proteins; Kim *et al.*, 1997; Ulmasov *et al.*, 1997a, 1999). The protein sequence of *LeIAA7* was deduced from a genomic clone since no corresponding cDNA clone was isolated.

Aligned amino acid sequences between domains II and IV (Figure 1) were used to calculate a distance-based tree with the Neighbor-Joining method (Saitou and Nei, 1987). The unrooted family tree shown in Figure 2 suggests that members of the *Aux/IAA* family can be grouped into five subfamilies. A sixth subfamily consisting of the ARF1-related proteins that are similar to the Aux/IAA family only in conserved regions III and IV (Ulmasov *et al.*, 1999) forms an outgroup. For most branches, the tree topology published by Abel *et al.* (1995) was confirmed in our analysis; however, the inclusion of many additional *Aux/IAA* sequences allowed us to classify some of the deeper branches as separate subfamilies. Bootstrap analysis

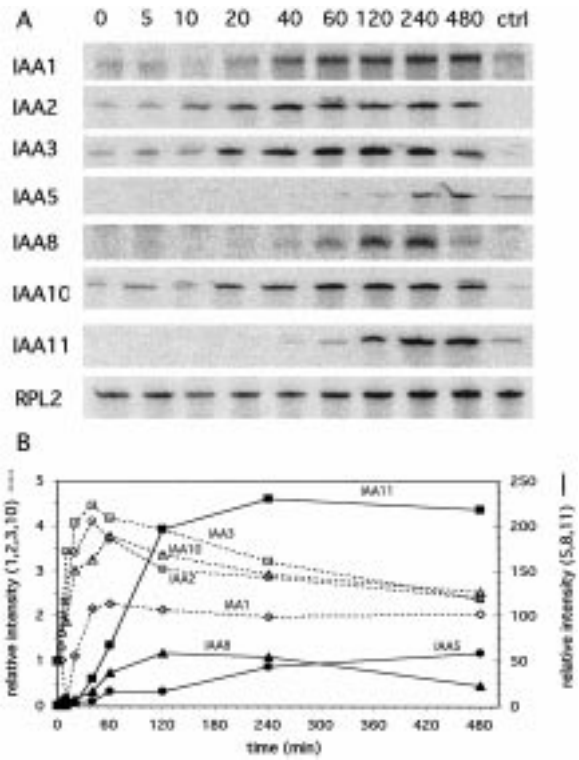


*Figure 4.* Kinetics of IAA-induced gene expression for representative *LeIAA* genes. A. 10 $\mu$g total RNA from 5–10 mm hypocotyl segments of 5-day old etiolated wild-type tomato seedlings treated for various times with 100 $\mu$M IAA were incubated with a radiolabeled antisense probe for a *LeIAA* gene and RPL2 control and RNase protection was detected as described for Figure 3. Lane numbers correspond to duration of treatment in minutes. The film exposure time for different genes was not equal, so transcript amounts cannot be compared between genes. A representative RPL2 control is shown. B. Relative changes in band intensities from panel A were determined densitometrically and normalized to the internal RPL2 control. The values plotted are relative to the 0 min time point and therefore represent fold induction over basal levels. Note the difference in scale between the left axis (*LeIAA1, 2, 3, 10*) and the right axis (*LeIAA5, 8, 11*).

of this phylogeny revealed that the same subfamilies were found in a consensus tree and were supported by bootstrap values of 69% to 99% (Figure 2). The only exception was subfamily III, which had a bootstrap value of 54%. The phylogenetic grouping presented in Figure 2 is supported by short sequence motifs that are characteristic of the respective subfamilies (Figure 1). An equivalent analysis of nucleic acid sequences from the same region yielded essentially the same results (data not shown). Several genes could not be assigned to specific subfamilies (*AtIAA15, 18,* and *20,* as well as *LeIAA2* and *8*) but emerged from short internal branches with low bootstrap values. The tomato genes isolated in this study thus represent all five phylogenetic subfamilies.

*Auxin induction in etiolated hypocotyls*

The effects of externally applied auxin on steady-state mRNA levels was studied in segments of etiolated hypocotyls to assess whether the *LeIAA* clones represent auxin-regulated genes. Of the eleven genes, ten could be visualized using RNase protection assays with probes derived from cDNA clones (data not shown). An mRNA corresponding to *LeIAA7* was not detected in etiolated hypocotyls by RNase protection assay using the genomic clone as a probe. A typical dose-response curve for auxin-induced expression of an *LeIAA* gene (*LeIAA11*) is shown in Figure 3. A probe corresponding to a constitutively expressed gene, *RPL2* (Fleming *et al*., 1993), was included in each RNase protection assay as a control for potential differences in RNA concentration. *LeIAA11* was maximally induced at 10 to 30 $\mu$M IAA (Figure 3), which is similar to the concentration dependence observed for *Aux/IAA* genes in other species (Theologis *et al*., 1985; Yamamoto *et al*., 1992; Abel *et al*., 1995). While reliable data could not be obtained for *LeIAA6* and *9*, which responded only weakly to auxin treatment, all other *LeIAA* genes tested showed similar auxin sensitivity, although the strong decline in transcript levels at high IAA concentrations was not observed for all of them (data not shown).

*Aux/IAA* genes from other plant species fall into different kinetic classes with respect to their response to auxin treatment (Abel and Theologis, 1996). It is reasonable to assume that rapidly responding genes are influenced by IAA in a more direct way and therefore are useful markers for the study of auxin-specific signal transduction events (Abel and Theologis, 1996). To test whether any of the tomato *LeIAA* genes fall into this category, we determined the changes in steady-state mRNA levels at various time points during incubation of hypocotyl segments with auxin. Individual *LeIAA* genes responded differently to IAA with respect to both the kinetics of mRNA accumulation and the maximal induction level (Figure 4 and Table 2). While some genes were expressed rapidly in response to IAA treatment and reached maximal expression levels in 60 min or less (*LeIAA1*, *2*, *3* and *10*), *LeIAA8* and *11* displayed intermediate kinetics. Others needed more than 2 h to reach maximal levels (*LeIAA4* and *5*). The first reproducible increases in mRNA abundance were observed within 10–20 min of auxin treatment.

*Table 1. IAA* clones isolated by PCR with degenerate primers B-DD2 and R-DD4.

| Gene | Clones isolated | | Fragment length (bp) | | Intron | GenBank |
|---|---|---|---|---|---|---|
| | genomic | cDNA | genomic | cDNA | length | accession number |
| *IAA1* | | + | 232 | 232 | 0 | AF022012 |
| *IAA2* | + | + | 284 | 208 | 76 | AF022013 |
| *IAA2′*[a] | + | + | 284 | 196 | 88 | |
| *IAA3* | + | + | 335 | 238 | 97 | AF022014 |
| *IAA4* | + | + | 418 | 301 | 117 | AF022015 |
| *IAA5* | + | + | ca. 770[b] | 349 | ca. 420 | AF022016 |
| *IAA6* | + | + | ca. 820 | 293 | ca. 530 | AF022017 |
| *IAA7* | + | | ca. 880 | 307 | ca. 570 | AF022018 |
| *IAA8* | | + | ca. 350 | 226 | ca. 120 | AF022019 |
| *IAA9* | | + | ca. 550 | 298 | ca. 250 | AF022020 |
| *IAA10* | | + | 1130 | 262 | 870 | AF022021 |
| *IAA11* | | + | ca. 430 | 232 | ca. 200 | AF022022 |

[a]Represents an alternative splice form of *IAA2*.
[b]Approximate values are based on size estimations from gel analyses.

This lag period is slightly longer than the 5–10 min reported for some *Arabidopsis AtIAA* genes (Abel *et al*., 1995) and may reflect either the physiological differences between *Arabidopsis* and tomato or our use of auxin-depleted hypocotyl segments rather than the intact seedlings used in the *Arabidopsis* study.

When assayed after 2 h of auxin treatment, the *LeIAA* genes displayed different levels of induction (Table 2). At this time point, when the majority of the gene family members were already maximally induced, most genes were found to have expression levels two- to six-fold higher than untreated controls. *LeIAA8*, *10*, and *11*, on the other hand, were induced by factors of ca. 27, 12, and 43, respectively. Similar differences were also found in the *Arabidopsis AtIAA* gene family (Abel *et al*., 1995). Taken together with the distribution in all of the phylogenetic subfamilies (Figure 2) and auxin concentration dependence (Figure 3), these results suggest that the *LeIAA* genes characterized in this study are a representative cross-section of *Aux/IAA* genes in tomato and are therefore suitable to investigate differentially regulated auxin responses in *dgt*.

### *Effects of the* diageotropica *mutation on auxin induction*

Basal levels of gene expression after depletion of endogenous IAA were similar for individual *LeIAA* genes in *dgt* and wild-type seedlings (Figure 5). In contrast, while the level of auxin induction was the same in both wild-type and *dgt* hypocotyl segments for some members of the gene family (*LeIAA1*, *2*, *3*), the auxin responsiveness of other genes (*LeIAA5*, *8*, *10*, *11*) was markedly reduced in mutant tissues (Figure 5, Table 2). The expression of *LeIAA4*, which was only slightly induced by IAA in wild-type tissue, was actually slightly repressed by auxin in *dgt* hypocotyl segments (data not shown). Notably, the two genes whose auxin responsiveness is most strongly reduced by the *dgt* mutation (*LeIAA8* and *LeIAA11*) are those which are normally most highly induced by auxin. However, even those genes whose auxin induction was strongly reduced in mutant hypocotyls showed some auxin responsiveness (Figure 5 and Table 2). This residual auxin responsiveness was similar for all affected genes (ca. 2- to 5-fold induction; Table 2) and was comparable to the low level of auxin induction typical of genes not affected by the *dgt* lesion.

Earlier studies (Mito and Bennett, 1995) have found that induction of the expression of *LeIAA1* (*LeAux* in their nomenclature) was strongly reduced (ca. 10-fold) in *dgt* hypocotyl segments, while we have found little or no effect of the mutation on this member of the gene family (Table 2; Figure 5). It is conceivable that the results reported by Mito and Bennett (1995) reflect the expression of several *LeIAA* genes since the northern blot method employed in that study may not have distinguished between the different gene family members.

*Table 2.* Expression characteristics of *LeIAA* genes in etiolated hypocotyl segments and intact seedlings.

| Gene[a] | Basal expression | Induction kinetics[b] | Auxin induction after 2 h[c] | | Endogenous expression in *dgt* v. WT |
|---|---|---|---|---|---|
| | | | WT | *dgt* | |
| *IAA1* | ++ | fast | 1.8 ± 0.1 (5) | 1.3 ± 0.7 (2) | similar |
| *IAA2* | ++ | fast | 2.4 ± 1.2 (5) | 1.4 ± 0.6 (3) | similar |
| *IAA3* | ++ | fast | 5.2 ± 1.6 (6) | 5.6 ± 1.4 (3) | similar |
| *IAA4* | +++ | slow | 1.6 ± 0.6 (6) | 0.6 ± 0.1 (3) | similar |
| *IAA5* | + | slow | 6.0 ± 2.6 (6) | 2.3 ± 0.3 (3) | similar |
| *IAA8* | + | medium | 26.5 ± 8.2 (6) | 2.6 ± 1.0 (3) | similar |
| *IAA10* | + | fast | 12.3 ± 5.5 (6) | 4.6 ± 3.1 (3) | reduced |
| *IAA11* | + | medium | 42.8 ± 11.0 (6) | 4.1 ± 2.3 (3) | reduced |

[a]*LeIAA7*, *LeIAA6*, and *LeIAA9* are not included due to a lack of transcript detection in etiolated seedlings (*7*) or a low level of expression that precluded accurate determinations (*6*, *9*).
[b]Time to reach maximal transcript levels after treatment with 100 $\mu$M auxin; 'fast': $\leq$1 h, 'medium': 1–2 h, 'slow': >2 h.
[c]Specifies mRNA abundance relative to basal for both wild-type (WT) and *dgt* hypocotyls, (mean ± SE). In parenthesis: number of independent determinations.

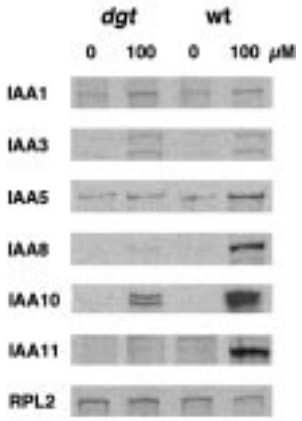


*Figure 5.* Induction of *LeIAA* genes by indole-3-acetic acid in wild-type and *diageotropica* hypocotyl segments. RNase protection assays were performed as described for Figure 3 with 15 $\mu$g total RNA from hypocotyl segments of 5-day old etiolated *diageotropica* or wild-type seedlings treated for 2 h with the indicated concentrations of IAA. The film exposure time for different genes was not equal, so transcript levels cannot be compared between genes. Densitometric analysis of this and additional experiments normalized to the internal RPL2 control is reported in Table 2.

Endogenous expression levels of the tomato *Aux/IAA* genes were examined in six regions of etiolated seedlings using multiplex titration RT-PCR (MTRP; Nebenführ and Lomax, 1998). This method allows estimation of transcript abundance based on the dilution step at which template concentration becomes limiting for successful PCR. The results obtained in this way cannot be compared quantitatively between different genes. However, they do allow a comparison of relative expression levels of a single gene between multiple samples and rapid analysis of a large number of samples from different tissues or conditions (Nebenführ and Lomax, 1998). Here, multiplex PCR with gene-specific primers targeting three different *LeIAA* genes and one control gene (*RPL2*) per reaction was run with a four-fold dilution series of cDNA derived from total RNA of different parts of the seedling as templates. Expression of most *LeIAA* genes tested was essentially the same in both wild-type and *dgt* seedlings (*LeIAA1*, *6* and *8*, Figure 6). Two genes, however, reproducibly displayed lower transcript levels in the mutant than in the wild-type plants; *LeIAA10* expression was reduced throughout the hypocotyl and root regions of *dgt* seedlings, while *LeIAA11* expression was much lower in *dgt* cotyledons and hypocotyls than in the same wild-type tissues. Surprisingly, although the expression of *LeIAA8* in response to exogenously applied IAA was strongly reduced in *dgt* hypocotyl segments, endogenous expression levels of *LeIAA8* were similar throughout mutant and wild-type seedlings (Figure 6).

## Discussion

We have isolated 10 new members of the *Aux/IAA* gene family of tomato (Figure 1). Together with the previously described *LeAux* gene (Mito and Bennett, 1995), this brings the family size in tomato to at least 11 genes. Based on the number of products resulting from PCR amplification of a genomic template, at

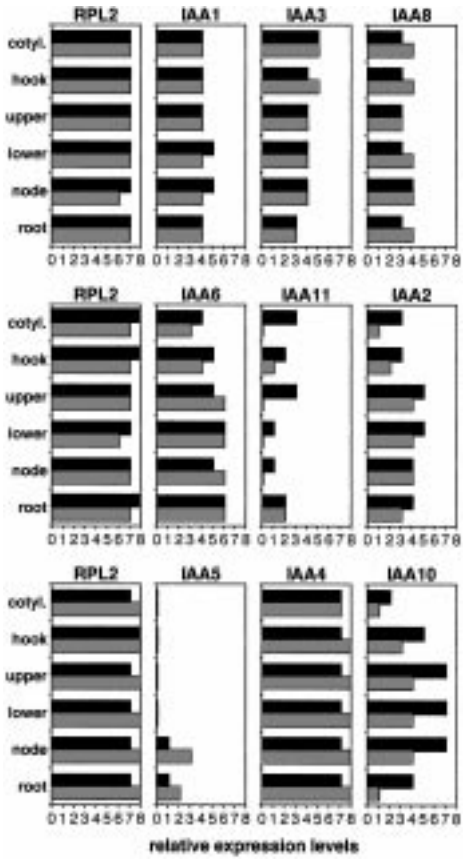


*Figure 6.* Tissue specificity of endogenous expression of *LeIAA* genes in intact etiolated wild-type and *diageotropica* seedlings. Seedlings of both *diageotropica* (gray bars) and its wild-type parent (VFN8, black bars) were grown for 5 days in the dark. Relative expression levels in various seedling tissues were determined by multiplex titration RT-PCR (MTRP) and reported as the dilution step at which template concentration became limiting. A representative experiment is shown. Seedling regions assayed were cotyledon, hook, upper and lower half of the hypocotyl, root-shoot node, and root. Each row of graphs represents one primer pool (Nebenführ and Lomax, 1998) where RPL2 was the internal control in each reaction. Numbers (0–8) below each graph refer to the number of four-fold dilutions of each template (e.g. 1 = 4-fold, 8 = 65,536-fold)

least two additional members of this gene family exist in tomato (data not shown). This number, as well as the phylogenetic analysis (Figure 2), demonstrates that the large number of *AtIAA* genes in *Arabidopsis* (Kim *et al*., 1997) is not unusual for dicotyledonous plants. The phylogenetic analysis presented here, which is based on partial amino acid sequences from the region between domains II and IV for each gene, closely resembles the phylogenetic tree published by Abel *et al*. (1995) that was based on full-length sequences of the genes available at that time. There are five distinct lineages within the classical Aux/IAA proteins that are connected by relatively short internal branches (Figure 2). The tomato sequences characterized here fall into all five *Aux/IAA* phylogenetic subfamilies and give a good representation of the structural diversity of *Aux/IAA* genes.

The concentration dependence for maximal induction of *LeIAA* gene expression (10–30 $\mu$M IAA; Figure 3) is also similar to what has been observed with the *Aux/IAA* genes in *Arabidopsis* (Abel *et al*., 1995) and other species (Theologis *et al*., 1985; Yamamoto *et al*., 1992). These values lie within the physiologically relevant concentration range of auxin (Cleland, 1995). Basal expression levels after auxin depletion of hypocotyl segments from etiolated seedlings varied widely, ranging from barely detectable (*LeIAA5*, *8*, and *11*) to high (*LeIAA4*). The *LeIAA* genes can be grouped into three kinetic classes based upon the time required to reach maximal expression after the onset of auxin treatment (Figure 4, Table 2). The 'fast' class reached maximal induction within 60 min or less (*LeIAA1*, *2*, *3*, and *10*), the 'medium' class was fully induced by 120 min (*LeIAA8* and *11*), and the 'slow' genes did not reach maximal mRNA abundance for at least 4 h (*LeIAA4* and *5*). Similar kinetic classes have also been described for the *Arabidopsis Aux/IAA* genes (Abel *et al*., 1995).

The induction of gene expression by two hours of auxin treatment varied on average from about 2-fold for *LeIAA1* and *LeIAA4* to greater than 40-fold for *LeIAA11* (Table 2). The fact that the class of genes that responded relatively rapidly included both weakly inducible genes (e.g. *LeIAA1*; ca. 2-fold induction) and strongly inducible genes (*LeIAA11*; >40-fold induction), suggests that distinct mechanisms regulate the expression of these genes.

Tomato seedlings with the *dgt* lesion have been reported to be insensitive to exogenously applied auxin with respect to hypocotyl elongation and ethylene production (Kelly and Bradford, 1986) and the sensitivity of root elongation to inhibition by exogenous IAA is reduced in the mutant (Muday *et al*., 1995). Auxin induction of the *SAUR* class of genes and the only previously examined tomato member of the *Aux/IAA* gene family is also dramatically reduced in *dgt* hypocotyls (Zurek *et al*., 1994; Mito and Bennett, 1995), but the expression of another auxin-regulated gene, *Lepar*, in response to auxin is not affected in *dgt* seedlings (Mito and Bennett, 1995). The significance of the latter finding was, however, not clear since *Lepar* encodes a glutathione *S*-transferase which responds to a wide range of agents and may have a primarily anti-

xenobiotic function (Abel and Theologis, 1996). Here, we show that within a single auxin-responsive gene family, only a subset of family members is affected by the *dgt* mutation. Specifically, IAA-induced expression of *LeIAA8* and *11* in etiolated hypocotyls was strongly reduced by the *dgt* lesion, whereas the mutation had little or no effect on the induction of *LeIAA1*, *2*, and *3* by auxin (Figure 5 and Table 2). The auxin induction of *LeIAA5* and *10* was moderately reduced in *dgt* hypocotyls. When relative endogenous expression levels were compared between wild-type and *dgt* seedlings, significant differences in transcript levels were found for only a few genes (*LeIAA10* and *11*, Figure 6), so the effect of $dgt^+$ appears to be restricted specifically to auxin responsiveness. Interestingly, one of the genes that showed greatly reduced auxin responsiveness in isolated hypocotyl sections (*LeIAA8*) had endogenous expression levels that were indistinguishable between *dgt* and wild-type seedlings (Figure 6). It is conceivable that *LeIAA8* is also regulated by factors other than auxin, and that these factors mask the auxin insensitivity in intact seedlings.

Those genes whose auxin response was most strongly reduced by the *dgt* mutation (*LeIAA8* and *11*) are also those most strongly induced by IAA in wild-type seedlings. It appears that only the most highly responding early genes are strongly affected by the *dgt* mutation. There is no apparent correlation between specific phylogenetic subfamilies and the division between those *LeIAA* genes that require $dgt^+$ function for IAA inducibility and those that do not.

The finding that auxin-induced expression is reduced by the *dgt* mutation in only a subset of *LeIAA* genes can be interpreted in several ways. It is conceivable that expression of all *LeIAA* genes is affected by the *dgt* lesion but that the methods employed in this study are not sensitive enough to detect these changes for weakly responding genes. However, *LeIAA3* and *5* are induced to similar levels by auxin in wild-type plants, but only *LeIAA5* is affected by the *dgt* lesion (Figure 5 and Table 2). It is also possible that those genes that respond normally to auxin in the mutant are induced in an indirect manner and do not represent genes in the direct path of auxin responses. This interpretation is not consistent with the observation that some early response genes (*LeIAA1*, *2*, and *3*) were not affected by the *dgt* lesion.

Alternatively, an intact $dgt^+$ gene product may be required for maximal induction of a specific subset of *LeIAA* genes. In this model, a $dgt^+$-independent mechanism is involved in the auxin-induced changes in transcript levels of the remaining genes. In this context, it is of interest that those *LeIAA* genes most strongly affected by the *dgt* lesion (*LeIAA8* and *LeIAA11*) are still weakly inducible by IAA in *dgt* hypocotyls (Table 2). This can be interpreted to mean that the *dgt* mutation is somewhat leaky or, alternatively, that more than one auxin-induced transduction pathway regulates the expression of these genes. The second interpretation implies that the $dgt^+$ gene product provides an additional auxin-dependent 'overdrive' function for some *LeIAA* genes, which would act in addition to a 'standard' induction mechanism common to all *LeIAA* genes. Loss of the enhancing function of the $dgt^+$ gene product would then result in similar induction levels for all genes of the family, a prediction born out by the results presented here (Table 2). A similar two-level regulation has been described for the promoter of a pea *Aux/IAA* gene, *PsIAA4/5*, where two regulatory elements act synergistically as auxin sensor (AuxRE A) and enhancer (AuxRE B) respectively (Ballas *et al*., 1995).

The results presented here confirm previous observations that the *diageotropica* mutation of tomato does not abolish auxin responsiveness completely (Muday *et al*., 1995; Rice and Lomax, 2000). For example, sensitivity to inhibition of gravicurvature by exogenously applied auxin is nearly identical in *dgt* and wild-type seedlings, indicating that auxin uptake, efflux, and at least one auxin receptor are functional in *dgt* (Rice and Lomax, 2000). Instead, the *dgt* lesion appears to diminish a subset of early auxin responses. The specific effect of the *dgt* mutation on the auxin-induced expression of a subset of *LeIAA* genes is in contrast to the *Arabidopsis* mutants *age1*, *axr1*, and *aux1*. In *age1* seedlings, all *AtIAA* genes tested showed a similar reduction in auxin responsiveness (Oono *et al*., 1998), whereas the *axr1* and *aux1* mutations had little effect on auxin-induced *AtIAA* gene expression (Abel *et al*., 1995). Similar to the semidominant *dgt* mutation, the dominant *axr2* mutation of *Arabidopsis* differentially affects auxin-induced expression of only some *AtIAA* genes (Abel *et al*., 1995). However, contrary to results found with the *LeIAA* genes in *dgt* seedlings, endogenous expression levels of the *AtIAA* genes are reduced in *axr2* plants. Therefore, *dgt* does not appear to be a homologue of any of these *Arabidopsis* genes. The differential effects on morphology and physiology caused by the various mutations also support this conclusion.

The differential effect of the *dgt* mutation on the expression of a subset of auxin-induced genes has the

potential to be used further to dissect the early events in auxin signal transduction in a manner similar to the analysis that has been performed with photoreceptor mutants (Neuhaus *et al*., 1993; Bowler *et al*., 1994). In particular, signaling molecules might be identified by co-injection into *dgt* hypocotyl cells together with a plasmid containing a reporter gene driven by the *LeIAA8* or *11* promoter. A strong response from the reporter construct would indicate that the co-injected signaling molecule acts downstream of the *dgt* lesion and would establish it as a second messenger involved in auxin signal transduction. The specificity of the *dgt* lesion within the induction cascade leading to increased expression of *LeIAA* genes confirms physiological data that the mutation does not affect some general aspect of auxin physiology, such as overall hormone metabolism or transport, and should allow the connection of specific gene products with downstream responses and phenotypes. While the exact function of the $dgt^+$ protein presumably will be determined only after the gene is cloned, the data presented here are consistent with models in which the $dgt^+$ gene product either regulates the auxin induction of a subset of *LeIAA* genes by specifically affecting a subset of signaling intermediates or, alternatively, is itself a signaling intermediate or transcription regulator.

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