AN ABSTRACT OF THE THESIS OF

Brandon M. Canfield for the degree of Bachelor of Science in BioResource Research presented on May 27, 2000. Title: Growth Regulator Interactions in the *diageotropica* Mutant of *Solanum lycopersicon*.

The *diageotropica* (*dgt*) mutation has been proposed to affect either auxin perception or responsiveness in tomato plants. Auxins and cytokinins are plant growth regulators known to interact with each other via a variety of mechanisms. Here, we report the interactions of auxins and cytokinins in VFN8 wild-type and *dgt* mutant seedling hypocotyl segments as relating to elongation, ethylene production, and expression of four members of the auxin regulated *Aux/IAA* gene family. The procedure differed from earlier studies in that the seedlings were germinated and grown in the presence of cytokinin, rather than treated with cytokinin just prior to auxin treatment. Conditional elongation and ethylene production for both wild-type and *dgt* were similar to those previously reported. With respect to auxin-induction, two of the genes tested for expression were found at levels comparable to those previously described and two were found at levels contrary to those previously described. To our knowledge, effects of cytokinin on expression of these *Aux/IAA* gene family members has not been previously examined. These results confirm that *dgt* plants have reduced sensitivity to auxin, that cytokinin reduces this sensitivity further, and that wild-type plants treated with cytokinin mimic only a subset of the effects of the *dgt* mutation on known auxin-responses.

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Growth Regulator Interactions in the *diageotropica* Mutant of *Solanum lycopersicon*

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Introduction

It has long been accepted that by varying levels and ratios of the plant growth regulators auxin and cytokinin, root, shoot, or callus tissue can be formed from cultured plant cells[1]. The observed interactions between these two hormones have been shown to be of either synergistic or antagonistic natures, such as the cytokinin-activation of an auxin-induced protein, related to the *cdc2* class of cyclin-dependent kinases, in tobacco pith explants[2], or the auxin-stimulation/cytokinin-inhibition of cell division during the initiation of lateral root primordia in higher plants[3]. Although physiological observations of these interactions abound, molecular explanations for the phenomena are lacking.

General hypotheses for the mechanisms underlying these interactions have been offered, including mutual controls of auxin/cytokinin pools, interactive controls of gene expression, or effects on some post-translational step[3]. To better investigate these possibilities, work has been carried out on the *diageotropica* (*dgt*) mutant of tomato (*Solanum lycopersicon*). This mutant has a reduced, but not completely abolished, sensitivity to auxin[4,5,6,7,8]. Attributed to the reduced auxin sensitivity of the single-gene, recessive mutant is a phenotype consisting of reduced apical dominance; stunting of root and shoot growth; dark green, hyponastic leaves; thin, rigid stems; and primary and adventitious roots that lack lateral root primordia, unless the root apex has been severely damaged[9,10].

In addition to the phenotypic abnormalities, *dgt* hypocotyl segments do not elongate in response to auxin and they demonstrate an inhibited response to auxin for the production of ethylene[4,6]. The growth of *dgt* callus tissue does not show normal dose response curves for either auxin or cytokinin, although evidence has been presented that the DGT gene product is not directly involved in cytokinin-signaling. Rather, it has been postulated that cytokinin action may depend on a functional auxin response[11]. Auxin-induced expression of two genes (*ACS3* and *ACS5*, previously reported as *BTAS2* and *BTAS3*[6]) encoding different isoforms of ACC-synthase (the key enzyme in ethylene

synthesis), a SAUR (Small Auxin Up-regulated RNA) gene, and *Aux/IAA* gene family members *LeIAA8*, *LeIAA10*, and *LeIAA11* have also been shown to be inhibited in *dgt* hypocotyl tissue[6,7,12]. However, 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*[8].

It should also be noted that shoot apices of *dgt* mutants contain normal levels of Indole-3-Acetic Acid[13] (IAA; the predominant native auxin), that the rate of polar auxin transport in *dgt* hypocotyls is essentially normal[14], and that *dgt* roots display no alterations in several transport-related phenomena[5]. This would indicate that the *dgt* mutation of tomato is likely to affect a specific step in auxin perception or signaling, providing an important tool for investigation of the molecular mechanisms underlying auxin-mediated processes[7].

In the presence of cytokinins, auxin-induced elongation of sunflower[15] and soybean[16] hypocotyl segments is inhibited, similar to what has been observed in *dgt*. Effects of cytokinin treatment on wild-type tomato hypocotyl segments include: reduction of auxin-induced elongation; inhibition of auxin-induced ethylene production; inhibition of auxin-induced expression of the *ACS3* gene[6]. In the same study, long term cytokinin treatment of wild-type plants resulted in phenocopies of untreated *dgt* morphologies. It is suspected that auxin stimulates expression of auxin-inducible genes through a DGT-dependent process leading to elongation and ethylene production, where cytokinin acts on only on a subset of these responses. In two alternative models, cytokinin either inhibits one branch of the auxin signal transduction pathway downstream from DGT, or it inhibits one of two separate DGT-dependent auxin signaling pathways[6]. It is also likely that DGT regulates only one of multiple auxin response pathways, based on the observations that auxin-induced expression of the *Lepar* gene[12] and the *LeIAA1, 2,* and *3* genes[7] were unaffected by the *dgt* mutation.

Here, we further examine the interactions of auxins and cytokinins in wild-type and *dgt* mutant tomato plants, with the ultimate goals of determining both the function of

the DGT gene product and the role of cytokinins in interfering with the auxin response pathway.

Much of our experimental design parallels that of the work carried out by Coenen et al.[6]. To test the possibility that cytokinins interfere with an early step in the auxin-response pathway, Coenen compared the effects of exogenously-applied cytokinins to those of the *dgt* mutation. Hypocotyl segments were treated with cytokinin in a preincubation similar in manner to that which we describe for the treatment of auxin (see: **Materials and Methods**). This raises the possibility that cytokinin levels adequate for interfering with an auxin-response may not have been present in the cells at the time of an early step in the pathway, or that any developmental differences resulting from exposure to constantly elevated levels of cytokinin may have been missed. Therefore, in our experimental design seedlings were germinated and grown in the presence of cytokinin.

Materials and Methods

Samples

Both wild-type and *dgt* tomato (*Solanum lycopersicon* Linn.) samples used in each experimental procedure were of the VFN8 background line, originally obtained from Dr. K. Bradford, University of California, Davis. Seeds used in the ethylene production and gene expression procedures came from field plants propagated in 1996 at the Oregon State University Botany Farm. Seeds used in the elongation procedure included a combination of those propagated at the Oregon State University Botany Farm in 1996 and 1998. Before germination, all seeds were soaked in 20% w/v household bleach for 10 minutes and then rinsed thoroughly.

Elongation

Seeds were sown onto one layer of 3 mm Whatman filter paper over four layers of Kimtowels (Kimberly-Clark Corp., Roswell, GA) saturated with either ddH_2O or 10 μM N^6 Benzyl-adenine (BA)_in plastic containers (24 x 30 x 9 cm; Pronee Plastics, Inc., Dixon, KY). Seeds were incubated for 4 to 6 days at 28°C in the dark.

Etiolated seedlings were harvested and treatments performed under fluorescent room light. Hypocotyl segments (1 cm) were removed below the hook of each seedling, and preincubated in 60 ml of 10 mM Potassium Phosphate ($K\text{-}P_i$) buffer for 2 h with gentle shaking to deplete endogenous IAA. After pre-incubation, 5 to 15 segments were transferred to plastic petri dishes containing 1 mM $K\text{-}P_i$ buffer and either 10 μl 95% ethanol (control samples) or 10 μl ethanol-based 10^{-1} M IAA (final concentration = 10^{-4} M IAA). Samples were immediately scanned (0 h) without size reduction or enlargement on a UMAX Astra 1220U computer scanner (UMAX Technologies, Inc., Fremont, CA), then incubated in light with shaking. Additional measurement scans were taken at 2.5 h and 24 h.

Computer scans were later analyzed and individual hypocotyl segments were measured using NIH Image 1.62 software (http://rsb.info.nih.gov/nih-image/). Growth and percent elongation were calculated according to differences in recorded lengths at time points 0 h and 24 h.

Ethylene Production

Hypocotyl segments were germinated, grown, and harvested as previously described. Segments were preincubated for 1 h and groups of either 15 or 30 segments were transferred to 10 ml crimp-top gas-tight scintillation vials containing 2 ml 10 mM K-

P_i buffer and either 20 µl 95% ethanol or 20 µl ethanol-based 10^{-1} M IAA (final concentration = 10^{-3} M IAA). Vials were sealed and incubated in light with shaking for 20 h to allow ethylene accumulation.

1 ml of the head space was removed from each sample and analyzed on a Shimadzu GC-14A gas chromatograph equipped with a Porapak N, 80/100 column (Alltech Associates, Inc., Deerfield, IL). Afterwards, the segments were dried for 72 h in a drying oven at 60°C. Dry weights were measured to normalize ethylene production.

Aux/IAA Gene Expression

Sample Preparation

Hypocotyl segments were germinated, grown, and harvested as described above, and pre-incubated for 1 h. Segments were then incubated in 25 ml fresh 10 mM K- P_i buffer and either 25 µl 95% ethanol or 25 µl ethanol-based 100 mM IAA (final concentration = 0.1 mM IAA) in light with shaking for 2 h. Segments were then divided into 1 g samples, wrapped in aluminum foil and quick-frozen in liquid nitrogen. Samples were then stored in a -80 °C freezer.

RNA extraction

Total RNA was extracted as described by Vernwood *et al.*[17]. Briefly, frozen samples were ground to a fine powder in a mortar and pestle in liquid nitrogen. 10 ml hot phenol extraction buffer (1:1 phenol:extraction buffer; 80°C) was added to the samples, which were then transferred to an RNase-free CF tube and vortexed. Following a brief incubation at 80 °C, 3 ml chloroform:isoAA (24:1) was added and samples were vortexed. Samples were centrifuged at 7000 rpm at 4°C for 15 min and the aqueous phase was transferred to new RNase-free CF tubes. 1 volume of 4 M LiCl was added and mixed by inversion. Total RNA was precipitated overnight at -20°C.

Samples were then thawed, vortexed, and centrifuged at 8000 rpm at 4°C for 20 min. The supernatant was removed, and the pellet dissolved in 2.5 ml DEPC-ddH_2O.

RNA was reprecipitated with 0.1 volume 3 M NaOAc, pH 5.2 and 2 volumes ice-cold 100% EtOH, mixed by inversion, then incubated at -80°C for 30 m. Following centrifugation (8000 rpm at 4°C for 15 min), pellets were rinsed with 7.5 ml ice-cold 70% EtOH and recentrifuged at 4°C for 5 min. Pellets were air-dried for 5 m and resuspended in 500 µl DEPC-H_2O. Approximate RNA concentrations were determined with a Beckman DU-64 spectrophotometer.

Reverse Transcription

Reverse transcription reactions were carried out essentially according to the protocol of the manufacturer (Promega Corp., Madison, WI). Briefly, the following was added to 500 µl microcentrifuge tubes: 1 µg RNA, 2 µl oligo $(dT)_{12\text{-}18}$ primer and DEPC-H_2O to a final volume of 17.75 µl. Samples were incubated in 70°C water bath for 5 min, cooled immediately on ice, and centrifuged for 30 s to collect solution at the bottom of the tube. To each was added 5 µl 5X reaction buffer, 1.25 µl 10 mM dNTP, and 1 µl M-MLV RT (H-) (Moloney Murine Leukemia Virus Reverse Transcriptase; 200 units/µl). Final volumes were brought to 25 µl with nuclease-free H_2O. Samples were mixed by gentle flicking and incubated for 60 min at 42°C. Samples were then spun for 1.5 min at 14k rpm in a microcentrifuge. 6 µl RNase was added and samples were heated in 37°C water bath for 20 min. 170 µl sterile H_2O and 1 volume phenol:chloroform (1:1) were added to each sample. Samples were vortexed, and incubated at room temperature for 5 min, then centrifuged at 14k rpm for 5 min. Aqueous layers were transferred to new 500 µl microcentrifuge tubes, and DNA was precipitated with 0.1 volume 3M NaOAc (pH 5.2) and 2 volumes 100% EtOH. Samples were incubated overnight at -80°C.

Following centrifugation (15 min at 14k rpm), samples were rinsed with 70% EtOH and allowed to air-dry for 15 min. cDNA pellets were resuspended in 30 µl sterile ddH_2O and concentrations were determined using a Beckman DU-64 spectrophotometer.

PCR Reactions

Gene sequences tested included four members of the *Aux/IAA* gene family: *LeIAA2, LeIAA3, LeIAA5,* and *LeIAA10*[7]. Each 25 µl PCR reaction contained 1 µM each gene specific forward and reverse primer, 200 µM dNTPs, 1X PCR reaction buffer, 2 mM $MgCl_2$, and 1 unit *Taq* polymerase. 250 ng cDNA was added from each sample treatment as template. A separate positive control contained 250 ng plasmid DNA which corresponded to each gene. Negative controls contained no DNA. PCR reactions were carried out on a Hot-Top Robocycler Gradient 96 (Stratagene, La Jolla, CA) with the following parameters: 35 cycles of 10 s at 94°C, 60 s at 62°C, and 90 s at 72°C. PCR products were loaded onto 1.5% agarose gels and run at 90-100 V for approximately 1 h, and visualized by UV transillumination and images digitally reproduced on a video copy processor (Mitsubishi Electronics, Cypress, CA).

Results

Previous research, carried out by Coenen *et al.*[6], found that short term (2h) incubation of wild-type tomato hypocotyl seedlings with 100 µM BA reduced their responsiveness to auxin with respect to both elongation and ethylene production. However, inhibition of those processes was not as complete as that produced by the *dgt* lesion. We germinated and grew the seedlings in 10 µM BA to see if prolonged, constant exposure to the hormone would produce a more complete inhibition than that previously reported. The 10 µM BA concentration was chosen from studies carried out by Coenen *et al.*[6], demonstrating it was the optimal concentration for phenocopying the *dgt* morphology in wild-type plants.

In addition to elongation and ethylene production, Coenen *et al.*[6] tested the *dgt* and wild-type responsiveness to auxin as indicated by expression levels of the *LeSAUR* gene and two isoforms of ACC synthase with or without cytokinin treatment. Auxin

increases the expression levels of the ACC synthase isoforms in a tissue specific manner[18], and expression of *SAUR* genes, whose biochemical function is not known[6], has been shown to be activated by auxin in soybean epicotyl segments within two to five m of application[19], making both excellent candidates to study the auxin response in tomato. However, given the recent isolation and characterization of partial clones representing eleven members of the *Aux/IAA* gene family in tomato[7], and given that only a subset of those genes are regulated by the DGT gene product, we investigated the effects of the different hormone treatments on the expression of the four of these genes.

Elongation

The length of seedling hypocotyl segments of both VFN8 wild-type and the *dgt* mutant, germinated and grown in either H_2O or 10 μM BA, and treated with either 100 μM IAA or EtOH, were compared following a 24 h incubation(Fig. 1 and 2). Data represent five independent experiments and were statistically analyzed using the 2-sample t-hypothesis test with significance levels based on $\alpha = 0.05$ (Table 1).

In segments from wild-type seedlings grown without BA, auxin-induced was observed. When wild-type was grown in BA, auxin-induced elongation was observed, but at a significantly lower level. In the absence of IAA, growth with BA did not significantly affect the elongation of the wild-type segments.

In *dgt* segments auxin-induced elongation was not observed in control or BA-grown samples. Interestingly, those segments grown in BA and treated with IAA actually showed a significant reduction in length relative to those segments grown in H_2O and not treated with IAA. In the absence of IAA, long term treatment with BA did not significantly affect the elongation of the *dgt* segments.

Ethylene Production

Ethylene content of the head space from sealed vials containing either VFN8 wild-type or the *dgt* mutant, which were germinated and grown in either H_2O or 10 μM BA, and treated with either 100 μM IAA or EtOH, was analyzed (see Fig. 3). Data represent five independent experiments and were statistically compared as previously described (Table 2).

In wild-type segments from plants grown in the absense of BA, auxin-induced ethylene production was observed. However, the presence of BA during development did not significantly alter the auxin-induced ethylene production in these samples. In the absence of IAA, growth in BA did not significantly affect the ethylene production of the wild-type segments.

Auxin-induced ethylene production in *dgt* segments was observed whether or not they were grown in BA. As observed in wild-type sample segments, BA did not affect ethylene production of uninduced *dgt* segments.

Aux/IAA Gene Expression

RNA expression levels of four members of the *Aux/IAA* gene family were evaluated using RT-PCR (Fig. 4 and Table 3). IAA treatment of wild-type segments decreased gene expression levels for *LeIAA2* (40 to 50%) and *LeIAA5* (10%), but increased gene expression for *LeIAA3* (3-fold) and *LeIAA10* (1.4-fold), independent of the presence of BA. Wild-type segments grown in BA and not treated with IAA demonstrated a further decrease in gene expression levels for *LeIAA2* (to 10% of basal levels) and for *LeIAA10* (to 30% of basal levels), while expression of *LeIAA5* was unchanged and *LeIAA3* increased to approximately half of auxin-induced levels.

Basal gene expression levels in *dgt* varied from wild-type for all sequences tested. A slight decrease was observed for *LeIAA5*, representing 90% of wild-type expression, while both *LeIAA2* and *10* demonstrated a more severe reduction to approximately 20% of those recorded for each gene in wild-type samples. In contrast, *LeIAA3* gene expression in *dgt* seedlings increased to over 3-fold from those observed in wild-type.

In seedlings grown in the absence of BA, IAA treatment of *dgt* segments decreased gene expression levels nearly in half for *LeIAA2* and *LeIAA10*, slightly increased gene expression levels for *LeIAA3*, and had no affect on *LeIAA5* expression. In seedlings grown in the presence of BA, IAA treatment of *dgt* segments decreased gene expression levels for *LeIAA2* (30%), for *LeIAA3* (50%), and for *LeIAA10* (60%), but had no affect on *LeIAA5* expression. In *dgt* segments untreated with IAA all demonstrated decreases in gene expression from their respective wild-type basal level: 80% decrease for *LeIAA2, 3,* and *10*; 60% decrease for *LeIAA5*.

Discussion

Effects of Cytokinin on Auxin-induced Elongation

To test the possibility that cytokinins interfere with an early step in the auxin-response pathway, Coenen et al.[6] compared the effects of cytokinins on auxin responsiveness in wild-type tomato seedlings to those in the *dgt* mutant seedlings. This was measured using known auxin-induced phenomena, including hypocotyl segment elongation and ethylene production. Segments were treated with BA two hours prior to treatment with IAA. Wild-type segments treated with BA were shown to partially phenocopy the *dgt* mutation, thus supporting the hypothesis that an early step in the auxin-response pathway is disrupted by cytokinin. However, the wild-type tomato segments treated with BA did not completely lose responsiveness to auxin, as has been shown in sunflower[15] and soybean[16] with regard to elongation.

We sought to replicate these experiments involving hypocotyl segment elongation and ethylene production with one difference: plant samples receiving cytokinin were germinated and grown in the presence of BA to test the hypothesis that prolonged exposure to BA would either allow higher concentrations to accumulate in the cells, or,

more likely, induce developmental changes which more dramatically affect auxin responsiveness.

When the seedlings were grown in 10 μM BA, we observed a significant reduction in auxin-induced elongation for wild-type hypocotyl segments. This is in agreement with the results previously reported for hypocotyl segments treated with 100 μM BA for 2 h. No additional reduction resulted from growing the seedlings in BA. Additionally, BA treatment did not significantly alter the reduced elongation response due to the *dgt* legion, which is comparable to what Coenen *et al.* observed[6].

Thus, the prolonged cytokinin exposure, as provided by germinating and growing etiolated seedlings in the presence of 10 μM BA, produced results similar to those obtained after a two hour treatment of 100 μM BA, with respect to the elongation auxin-response. The incomplete elimination of the auxin-induced elongation in tomato by cytokinin, in contrast to sunflower[15] and soybean[16], could be due to a variance in plant background.

Effects of Cytokinin on Auxin-induced Ethylene Production

We found no significant effects on ethylene production for auxin-induced wild-type segments grown in cytokinin. This is in contrast to observations by Coenen *et al.*[6], who found that treatment with 100 μM BA significantly reduced the magnitude of auxin-induced ethylene production in wild-type segments and thus produced partial phenocopies of the *dgt* mutation. This difference could be due to increased levels of IAA used in our treatments, which were a tenfold increase over those previously reported. The possibility exists that such high auxin levels were able to overcome any putative cytokinin effect previously observed.

Also, we observed that *dgt* segments displayed slight, yet significant, increase in auxin-induced ethylene production in the absence of cytokinin. However, since the increase was very minimal with a significance value very near the $\alpha = 0.05$ level, we

hesitate to challenge previously reported results until further investigation can be completed.

Effects of Cytokinin on the Auxin-induction of *Aux/IAA* Gene Expression

After finding that cytokinin treatment phenocopied *dgt* effects on plant morphology as well as on various physiological target reactions, including hypocotyl segment elongation, proton secretion, and ethylene synthesis, Coenen *et al.*[6] concluded that cytokinin and DGT may control a shared set of auxin responses. To determine if this putative control were at the transcriptional level, expression levels of *LeSAUR*, *ACS3*, and *ACS5* genes were measured[6]. In *dgt* hypocotyl segments, auxin-inducibility of *LeSAUR* and *ACS3* transcripts was markedly reduced and induction of *ACS5* transcripts by auxin was completely absent. In wild-type segments, transcripts of both *ACS* isoforms increased in response to auxin. Addition of cytokinin strongly inhibited the auxin-response for *3* and not effecting *LeSAUR* or *ACS5* at all. Thus, cytokinin treatment partially mimicked the effects of the *dgt* mutation on the expression of *ACS3*, but not on *LeSAUR* or *ACS5*.

Recent studies increased the *Aux/IAA* gene family to at least 11 members in tomato[7] and at least 25 members in *Arabidopsis*[20]. The *Aux/IAA* proteins have been shown to form homo- and heterodimers, and to interact with the related group of ARF1 (Auxin Response Factor 1) -like transcription factors[20]. These characteristics, together with the rapid induction of mRNA and the short half-life of proteins make the *Aux/IAA* gene products excellent candidates for signaling intermediates in auxin responses[21], and thus suitable for the study of auxin-induction.

Of the 11 tomato *Aux/IAA* gene clones previously isolated and characterized, we determined the level of expression for family members *LeIAA2*, *3*, *5*, and *10*. A prior study found that the *dgt* mutation had little or no effect on the induction of *LeIAA2* and *3* by auxin and that the auxin induction of *LeIAA5* and *10* was moderately reduced in *dgt*

hypocotyls[7]. Significant decreases in *dgt* endogenous expression levels, relative to wild-type, was found in *LeIAA10*, but not in *LeIAA2*, *3*, or *5*[7].

Regarding expression of *LeIAA2*, we observed an auxin-induced inhibition in both wild-type and *dgt* segments in the absence of BA. We also saw a fivefold decrease in *dgt* basal expression from wild-type segments. In both wild-type and *dgt* segments, we observed a strong decrease in basal expression when grown in BA (90% and 80%, respectively), and a slight increase in auxin-induced expression when grown in BA (5% and 10%, respectively). This would indicate that cytokinin affects the expression of the *LeIAA2* gene independent of the *dgt* lesion. Since this experiment was not replicated, and in light of the contradictory nature of our results to those previously reported[7], further study must be performed to better understand the putative role of cytokinin in expression of *LeIAA2*.

Basal expression levels of *LeIAA3* were increased in *dgt* hypocotyl segments as compared to wild-type, with little or no effect of IAA on *dgt* segments. However, auxin-induction increased gene expression in wild-type. Cytokinin reduced auxin sensitivity in *dgt*, but not in wild-type. Again, these observations differ from previous work[7], and should be further examined to understand the effect of cytokinin in expression of *LeIAA3*.

For the expression of *LeIAA5*, we observed no auxin-induced response of either wild-type or *dgt* segments and no difference between basal levels of expression in wild-type and *dgt*. These results are in partial agreement with the observations of the previous study, which proposed that the absence of auxin-induced expression was due to an increased treatment period necessary. *LeIAA5* required at least four hours of incubation for maximal expression levels[7]. The expression level in *dgt* segments grown in BA, but not induced with IAA, were approximately half of the basal level. Except this reduction, neither hormonal treatments nor the *dgt* mutation had an effect on the expression levels of *LeIAA5*. The implications of this could prove valuable in the continuing study of the interactions between auxin, cytokinin, and DGT, and obviously deserves replication. It should be noted that expression of *LeIAA5* is missing from the gel presented in Fig. 4. However, the remaining banding pattern confirmed levels observed in prior trials (not

reported here), and the intensity levels reported in Table 3. are relative to those from said trials.

Regarding expression levels of *LeIAA10*, we observed a fivefold decrease in *dgt* basal expression from wild-type segments. Auxin-induced expression levels were increased for wild-type and decreased for *dgt*. While BA decreased basal expression levels for both wild-type and *dgt* segments, this affect was nearly masked by auxin-induction in wild-type. *dgt* segments displayed a reduction in auxin-induced expression levels when grown in BA. With no replications, the results of this experiment more or less agrees with the earlier study[7]. With this in mind, specific attention should be paid to the segments when grown in the presence of cytokinin and not induced with auxin, both wild-type and *dgt* demonstrated a near 5-fold decrease in expression levels relative to their respective basal expression (however, no speculation as to relevance of these observations will be made until the experiment is replicated). It should be noted that in this trial, the *IAA10* plasmid positive control failed to yield a band. Yet the sample banding pattern matches the expected product size and is therefore accepted as a reflection of expression level[7].

Summary

Our results support the notions that the *dgt* mutant has a reduced sensitivity to auxin upon treatment with cytokinin and that cytokinin inhibits the auxin response mechanism in wild-type plants as well.

Expression studies in *dgt* seedlings demonstrate that only a subset of the *Aux/IAA* genes are affected by the lesion and suggest that the DGT gene product is involved in an early step in the regulation of gene expression by auxin[7]. Our results confirm that cytokinin treatments of wild-type hypocotyl segments mimic only a subset of the effects of the *dgt* mutation on known auxin-responses and that the limited effects of cytokinin on DGT-dependent auxin responses can be explained by either cytokinin acting on a branch of auxin-signal transduction downstream from DGT or by the existence of two separate,

DGT-dependent pathways of auxin signaling[6]. Results obtained from samples germinated and grown in the presence of cytokinin seemed to not differ from those reported from samples treated for only two hours[6], indicating that cytokinin does not interact with the auxin response pathway in a developmental manner.

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Table 1. Significance analysis of the *dgt* and *VFN8* conditional elongation trials.

	growth[#] mean ± st. error[@]	percent change mean	*		growth[#] mean ± st. error[@]	percent change mean	*
dgt **+H_2O**	0.05 ± 0.06	0.55	b	**VFN8 +H_2O**	-0.03 ± 0.04	-0.34	x
dgt **+H_2O +IAA**	-0.01 ± 0.08	-.16	a,b	**VFN8 +H_2O +IAA**	0.58 ± 0.06	5.87	z
dgt **+BA**	0.12 ± 0.04	1.17	a,b	**VFN8 +BA**	0.03 ± 0.03	0.25	x
dgt **+BA +IAA**	-0.09 ± 0.08	-0.85	a	**VFN8 +BA +IAA**	0.43 ± 0.04	4.31	y

[#] (mm)

* Significantly distinct groups based on 2-sample t-test, $\alpha = 0.05$; significance calculated from both growth and percent change produced equivalent patterns.

[@] n = 9-27

Included are the mean growths and percent changes in length followed by standard errors for each sample treatment. The fourth column shows that the only significant differences among the *dgt* samples were between *dgt* grown in water and *dgt* grown in cytokinin and treated with auxin. The eighth column shows that wild-type grown in water and treated with auxin had a significantly larger growth response than wild-type grown in cytokinin and treated with auxin, which itself had a significant growth response over wild-type not grown in water or cytokinin and not treated with auxin.

Table 2. Significance analysis of the *dgt* and *VFN8* conditional ethylene production trials

	ethylene production[#] mean ± st. error[@]	*		ethylene production[#] mean ± st. error[@]	*
***dgt*+H_2O**	0.69 ± 0.09	a	**VFN8+H_2O**	2.04 ± 0.73	x
***dgt*+H_2O+IAA**	1.51 ± 0.3	b,c	**VFN8+H_2O+IAA**	6.42 ± 1.1	y
***dgt*+BA**	0.70 ± 0.04	a	**VFN8+BA**	1.83 ± 0.58	x
***dgt*+BA+IAA**	1.45 ± 0.34	a,b	**VFN8+BA+IAA**	5.43 ± 1.27	y

[#] (nl ethylene/ 10 ml gas/ mg dry weight).

* Significantly distinct groups based on 2-sample t-test, $\alpha = 0.05$.

[@] n = 5-14

Included are the mean ethylene production values for each sample treatment and respective standard errors. Column three shows that basal *dgt* ethylene production is not affected by growth in cytokinin. Auxin-induced ethylene synthesis is also not affected by growth in cytokinin in *dgt*. Column six shows significant auxin-induced ethylene production in wild-type grown in water or cytokinin, and that there is no difference between them. Wild-type basal levels did not differ when grown in water or cytokinin.

Table 3. Relative intensity levels of *Aux/IAA* gene expressions

	VFN8 +H_2O	VFN8 +H_2O +IAA	VFN8 +BA	VFN8 +BA +IAA		*dgt* +H_2O	*dgt* +H_2O +IAA	*dgt* +BA	*dgt* +BA +IAA		*dgt*+H_2O/ VFN8+H_2O
LeIAA 2	1	0.5	0.1	0.6		1	0.5	0.2	0.7		0.2
LeIAA 3	1	3.0	1.8	3.1		1	1.2	0.2	0.5		3.4
LeIAA 5	1*	0.9*	0.9*	0.9*		1	1	0.4	1.0		0.9*
LeIAA 10	1	1.4	0.3	1.3		1	0.6	0.2	0.4		0.2

* based on trends observed in previous experiments (not reported here).

Wild-type and *dgt* band intensities were normalized to their respective basal levels for each gene. The last column shows the relation of the basal *dgt* level to the basal wild-type level for each gene. Data reported are from one trial each per gene, and were not replicated.

Figure 1. Hypocotyl segment conditional growth time course. Growth of 10 mm seedling hypocotyl segments at time points 0, 2.5, and 24 h after having been germinated and grown 4-6 days in or out of the presence of 0.1 mM BA, preincubated for 2 h in 10 mM K-Pi buffer and treated with or without 100 μM IAA. Only lengths measured at 24 h were analyzed for comparisons, although the 2.5 h data is included for visualization of the response. Error bars show standard errors from five independent experiments.

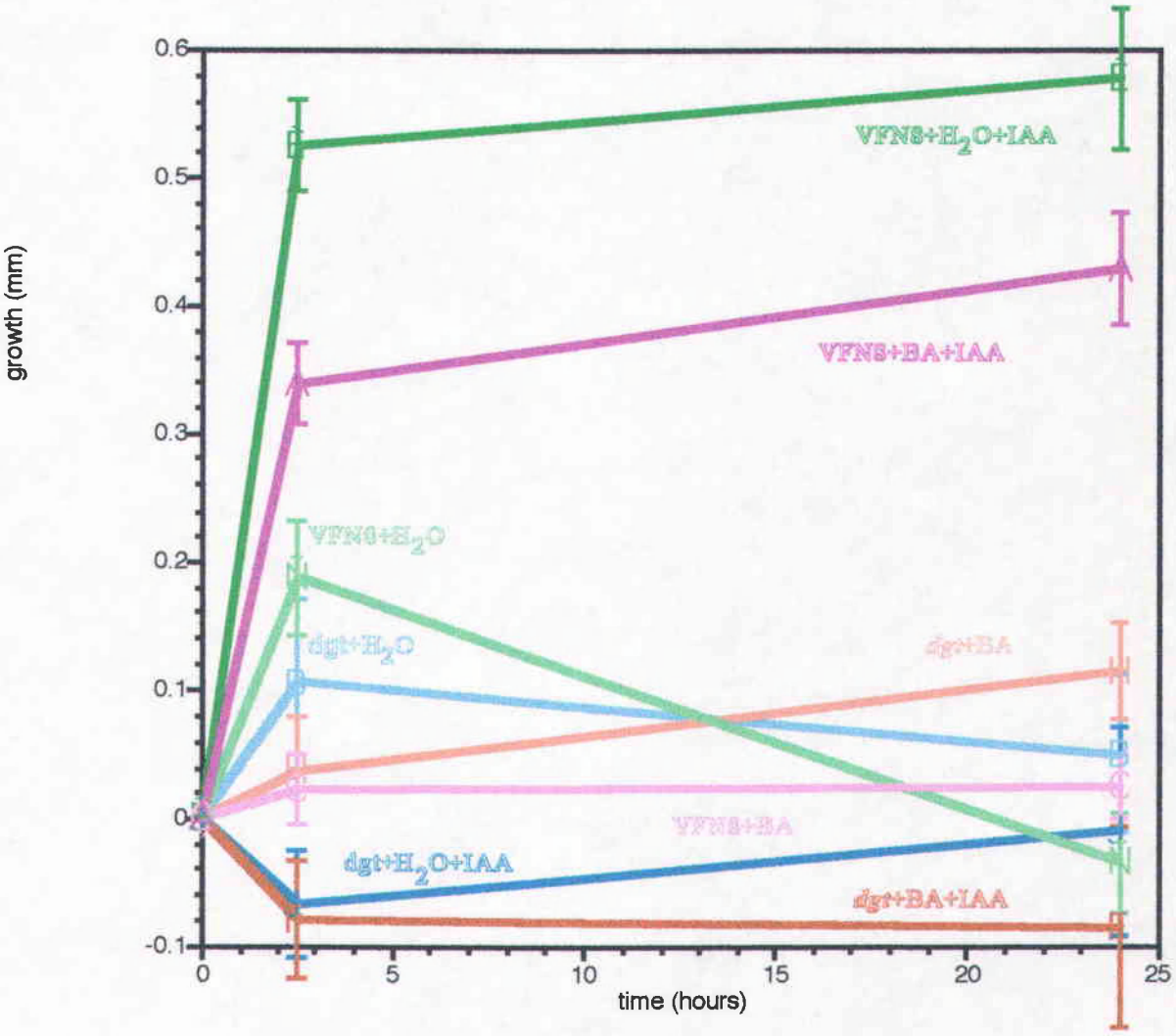


Figure 2. Hypocotyl segment conditional growth as a percent of change time course. Percent of change of 10 mm seedling hypocotyl segments at time points 0, 2.5, and 24 h after having been germinated and grown 4-6 days in or out of the presence of 0.1 mM BA, preincubated for 2 h in 10 mM K-Pi buffer and treated with or without 100 µM IAA. Only lengths measured at 24 h were analyzed for comparisons, although the 2.5 h data is included for visualization of the response.

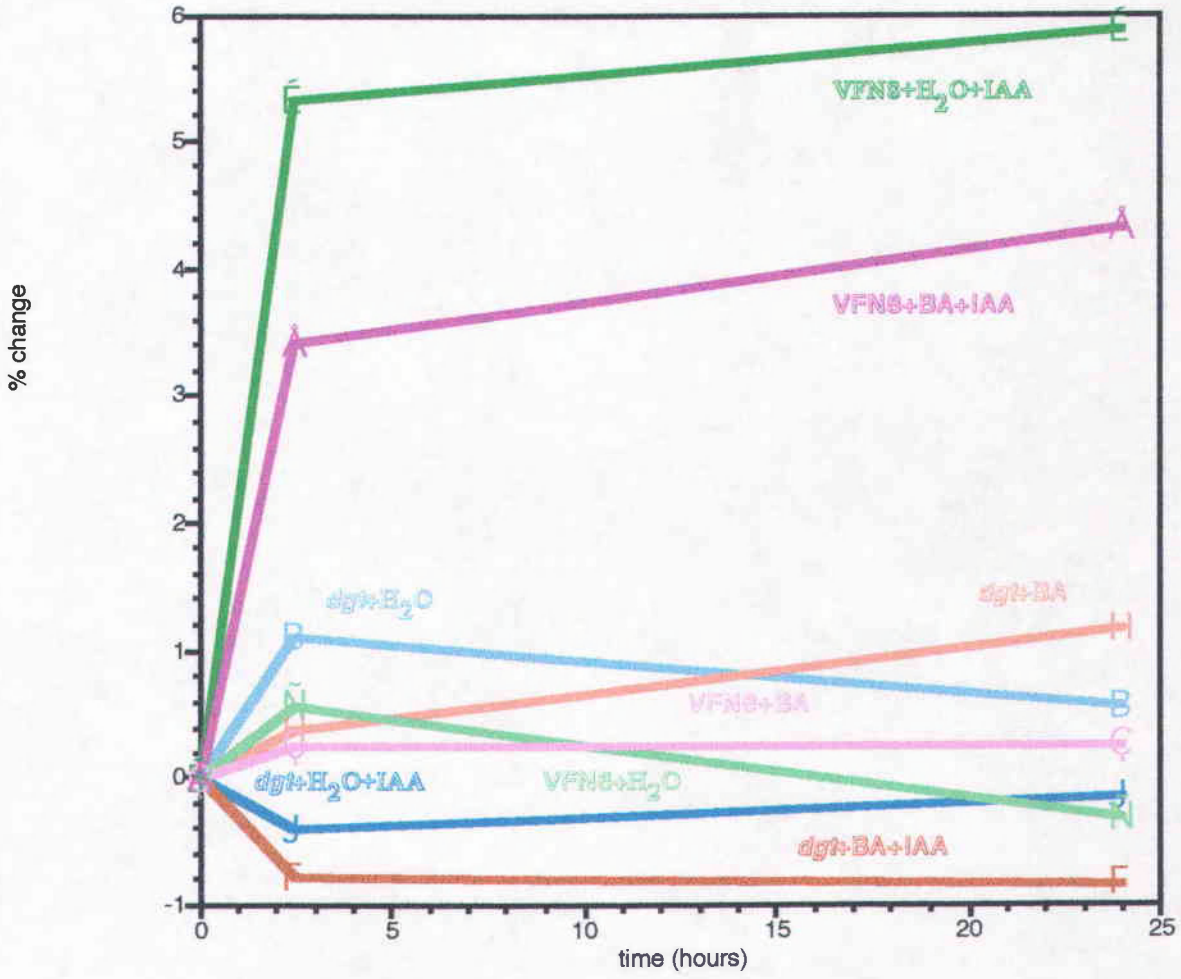


Figure 3. Hypocotyl segment conditional ethylene production. Ethylene content of the gas phase from sealed vials containing 10 mm seedling hypocotyl segments germinated and grown for 4-6 days in or out of the presence of 10 μM BA, preincubated for 1 h in 10 mM K-Pi buffer and treated with or without 1 mM IAA after 20 h. Error bars show standard errors from five independent experiments.

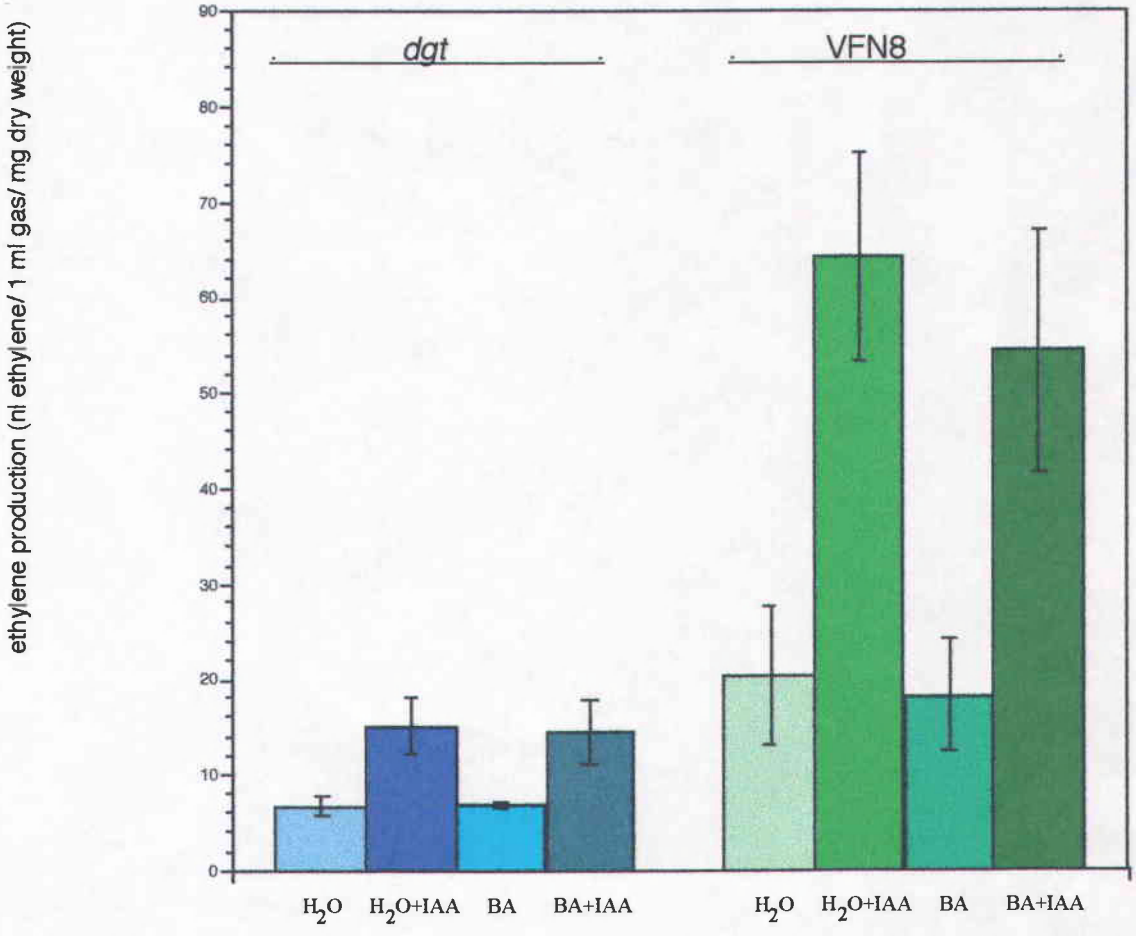
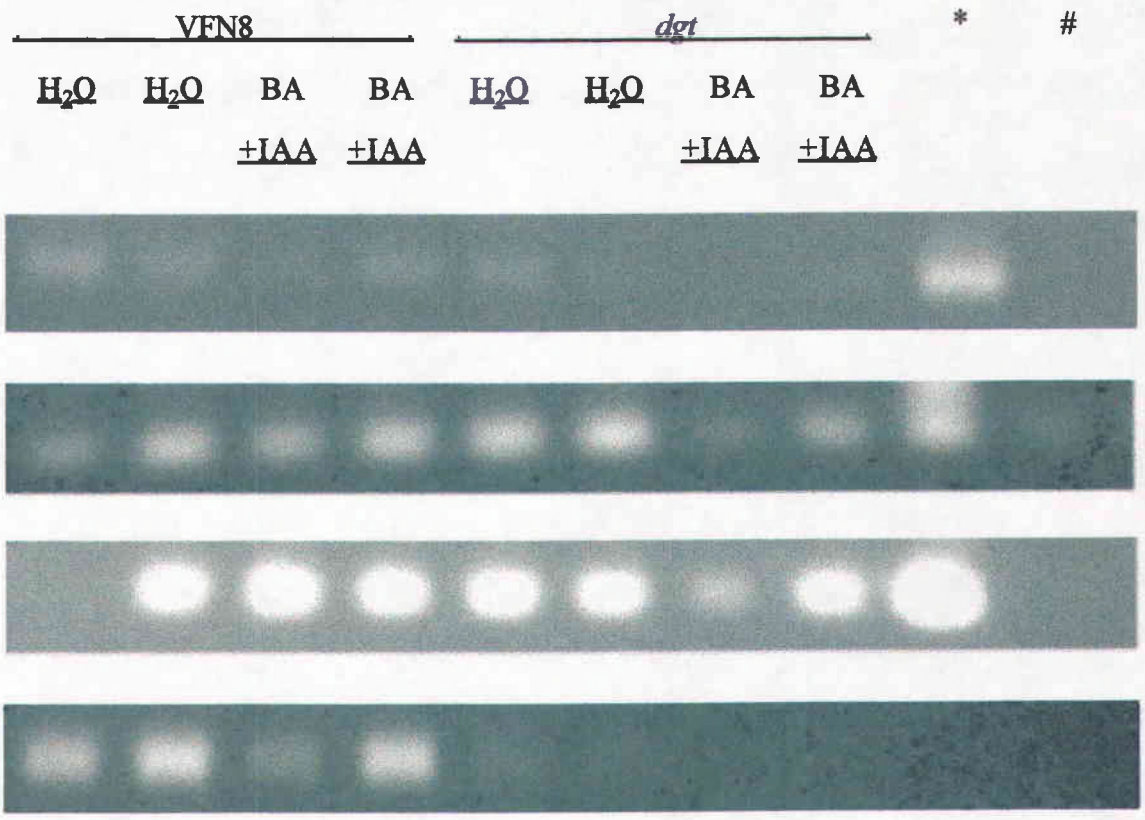


Figure 4. Hypocotyl segment conditional expression of *Aux/IAA* genes. Expression of *LeIAA2*, *3*, *5*, and *10* genes in 10 mm seedling hypocotyl segments germinated and grown 4-6 days in or out of the presence of 10 μM BA, preincubated for 1 h in 10 mM K-Pi buffer and treated with or without 0.1 mM IAA for 2 h. Rows correspond to gene tested for; gel lanes correspond to sample conditions, with the second to last lane representing the plasmid positive control, and the last lane the master mix negative control. Gels are from the top to bottom, respectively, *LeIAA2*, *LeIAA3*, *LeIAA5*, and *LeIAA10*.



* plasmid positive control

\# negative control