Plant Physiol. (1986) 82, 713–717
0032-0889/86/82/0713/05/$01.00/0

Insensitivity of the *Diageotropica* Tomato Mutant to Auxin[1]

Received for publication December 31, 1985 and in revised form June 6, 1986

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ABSTRACT

The sensitivity of excised hypocotyl segments to indoleacetic acid (IAA) in two assays, ethylene production and elongation, was determined in the ethylene-requiring tomato (*Lycopersicon esculentum* Mill.) mutant, *diageotropica* (*dgt*), and its isogenic parent, cv VFN8. Endogenous (uninduced) ethylene synthesis rates were slightly lower in *dgt* hypocotyls than in VFN8 hypocotyls. Ethylene production was essentially unaffected by IAA in *dgt*, but was stimulated up to 10-fold by 10 micromolar IAA in VFN8. Elongation of *dgt* hypocotyls was also insensitive to concentrations of IAA as high as 100 micromolar, as compared to significant elongation of VFN8 hypocotyls in response to 0.1 micromolar IAA. A range of IAA analogs active in VFN8 was also ineffective in stimulating elongation of *dgt* hypocotyls, suggesting that the differences were not due to rapid metabolism of IAA by *dgt* tissues. Auxin-induced elongation of VFN8 hypocotyls was unaffected by 2,3,5-triiodobenzoic acid and naphthylphthalamic acid, indicating that polar auxin transport was not a factor in these experiments. Exogenous and auxin-induced ethylene had no effect on the elongation respone of either genotype, nor did exogenous ethylene restore the sensitivity of *dgt* hypocotyls to IAA. Despite their apparent insensitivity to auxin, *dgt* hypocotyls elongated dramatically and synthesized ethylene rapidly in response to 1.2 micromolar fusicoccin. These results suggest that the primary effect of the *dgt* mutation is to reduce the sensitivity of the tissue to auxin. As altered regulation of ethylene synthesis is only one symptom of this fundamental deficiency, *dgt* should more properly be considered to be the auxin-insensitive tomato mutant.

The *diageotropica* tomato mutant is characterized by horizontal shoot and root growth, thin stems, hyponastic leaves, and lack of lateral roots (27, 28). The genetic lesion is a mutation in a single gene of the parent variety, VFN8 (26). Application of exogenous ethylene could phenotypically normalize the geotropic response and some morphological features of *dgt*[2] plants (5, 27, 28), and IAA was less effective in stimulating ethylene synthesis in *dgt* stem sections as compared to VFN8 (27). These observations led Zobel (27) to propose that *dgt* is an ethylene-requiring mutant because the failure of auxin to induce ethylene synthesis results in low endogenous levels of ethylene. In subsequent studies, however, endogenous (uninduced) ethylene synthesis rates were not always lower in *dgt* tissues than in normal plants (3, 5). In addition, the induction of ethylene synthesis in *dgt* by wounding or anaerobiosis was apparently normal (3, 5). Thus, the fundamental genetic lesion of *dgt* does not simply result in an inability to synthesize ethylene. Rather, the common feature of the studies cited is the very poor effectiveness of IAA in stimulating ethylene synthesis rates in *dgt* stems (27), roots (5), and petioles (3) compared to tissue from VFN8.

The failure of auxin to induce ethylene synthesis in *dgt* tissues could be due to altered regulation of the expression of the gene(s) encoding the rate-limiting enzyme, ACC synthase (3, 24). Alternatively, the ineffectiveness of IAA in inducing ethylene synthesis may be only one manifestation of a more general insensitivity of *dgt* plants to IAA. It is important to distinguish between these two possibilities. If only the induction of ACC synthase is abnormal, the mutant may be useful in identifying the mechanism of selective gene expression regulated by auxin (4, 22). On the other hand, if *dgt* tissues exhibit a general insensitivity to auxin, the mutant may represent a potentially powerful tool for identification and characterization of the receptors presumably responsible for auxin action (9, 16, 18). We have, therefore, investigated the sensitivity of *dgt* to auxin in an auxin-stimulated process, hypocotyl elongation, which we show is independent of auxin-induced ethylene synthesis.

MATERIALS AND METHODS

Plant Material. Tomato (*Lycopersicon esculentum* Mill.) seeds of the *dgt* mutant and its isogenic parent, cultivar VFN8 (obtained from Dr. C. M. Rick of this department), were surface sterilized for 20 min in 1% NaOCl solution (20% bleach). One-hundred seeds were sown on moist blotter paper in a covered 4 × 4 cm plastic box at 27°C in the dark. After 109 to 115 h, a 6 or 10 mm segment was excised from the hypocotyl with a double razor blade cutter. Cuts were made as close to the hook as would yield straight segments. Immediately after cutting, segments were floated on 2.5 mM KH_2PO_4 (pH 5.2) for 1 to 3 h. For treatments with ethylene synthesis or action inhibitors, segments were pretreated for 1 h with the inhibitor at the desired concentration in the above solution.

Hypocotyl Elongation Assay. Twenty 6-mm segments were selected at random and floated on 20 ml of a basal medium containing 2.5 mM KH_2PO_4, 2.5 mM KCl, 1 mM $Ca(NO_3)_2$, and 3% sucrose (pH 5.2) in a 200 ml beaker. Chemicals were added to the basal medium in small volumes from concentrated stock solutions stored in the freezer or refrigerator. IAA solutions were prepared daily. The pH was readjusted, if necessary, after compounds were added to the basal medium. Segments were incubated for 10 h on a shaker at 27°C under laboratory light. Segment lengths were then measured to within 0.1 mm with a dissecting microscope equipped with a stage micrometer. Each data point represents the mean of 15 to 20 segments, since damaged and severely bent segments were discarded. Each experiment was performed at least twice. The final lengths have been normalized to percentages of the respective controls.

Elongation was measured in the presence of ethylene as above

[1] Supported in part by National Science Foundation Grant No. DMB-8408857.

[2] Abbreviations: *dgt*, *diageotropica*; ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; pCPA, para-chlorophenoxyacetic acid; oCPA, ortho-chlorophenoxyacetic acid; FC, fusicoccin; αNAA, α-naphthaleneacetic acid; βNAA, β-naphthaleneacetic acid; NPA, naphthylphthalamic acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; TIBA, 2,3,5-triiodobenzoic acid.

with the following modifications. Segments were placed in 50-ml flasks containing 10 ml of solution and the flasks were then sealed with serum caps. To trap evolved CO_2, a plastic center well containing a small piece of fluted filter paper saturated with 150 μl of 5 N NaOH was inserted through the serum cap. Due to a high gas phase volume to tissue weight ratio (about 500 ml/g fresh weight), O_2 depletion was not anticipated. This was confirmed by measuring the O_2 concentration in the gas phase by GC during the latter half of the experiment. The O_2 concentration was never less than 15%. Aliquots of known concentrations of ethylene were injected through the serum caps after removing the same volume of air from the beaker. The ethylene concentration of the gas phase was confirmed by GC at the beginning and during the latter half of the experiment. In cases where the tissue would not be expected to synthesize significant quantities of ethylene, the second measurement was usually between 85 to 110% of the first, and never varied by more than 25%. Controls were treated similarly except that no ethylene was added to the bottles.

Ethylene Measurement. Fifteen 10-mm segments were chosen at random and floated in 10-ml bottles on 1 ml of elongation assay solution. The bottles were incubated on a shaker at low speed to ensure aeration. Bottles were left open for 2 h, after which they were sealed with a serum cap. The ethylene concentration of the gas phase was measured by GC 3 h after sealing. Background ethylene (measured in the absence of tissue) has been subtracted from all ethylene synthesis rates reported. The CO_2 and O_2 concentrations of the gas phase were measured 3.5 to 4 h after sealing to determine the extent of CO_2 accumulation and O_2 depletion during the treatment time. Both treated and untreated samples of VFN8 and *dgt* hypocotyls were measured at random. The O_2 concentration was never lower than 15% and the CO_2 concentration was 0.5 to 2%. CO_2 promoted the conversion of ACC to ethylene in rice and tobacco, with a half-maximal velocity close to ambient CO_2 concentrations (6). Thus, the CO_2 concentration should have been close to optimal for ethylene synthesis.

RESULTS

The rate of ethylene synthesis was maximal in VFN8 hypocotyls at 10 to 100 μM IAA (Fig. 1). Only a slight stimulation of ethylene production was observed in *dgt* hypocotyls even at 100 to 500 μM IAA (Fig. 1). Uninduced ethylene synthesis rates were 31% lower in *dgt* than in VFN8 hypocotyls (0.82 *versus* 1.18 nl g^{-1} h^{-1}, respectively; F test significant at $P < 0.01$ for two experiments with three replicates each).

Stimulation of elongation of VFN8 hypocotyls was detectable at 0.1 μM IAA and saturated typically at 1 to 10 μM IAA (Fig. 2). The most rapid growth of VFN8 hypocotyls occurred during the first 2 to 3 h after treatment, but continued throughout the 10 h incubation time (data not shown). Hypocotyls from *dgt* seedlings generally did not elongate after treatment with up to 100 μM IAA, but sometimes elongated in response to 500 μM IAA (Fig. 2). The results presented in Figure 2 are typical of two experiments. In some experiments, however, small but statistically significant increases in growth were observed at concentrations below 500 μM IAA, while in other experiments no growth occurred at any IAA concentration.

The ability of exogenous ethylene to restore normal geotropic orientation in *dgt* (5, 27) suggests that ethylene may influence auxin sensitivity. This was tested by determining whether exogenous ethylene could restore the ability of *dgt* hypocotyls to grow in response to IAA. No growth was observed in *dgt* hypocotyls in response to 10 μM IAA in the presence of up to 2 μl/L of exogenous ethylene (Fig. 3). Ethylene also had no significant effect on growth of either *dgt* or VFN8 hypocotyls in the absence of auxin (Fig. 3). VFN8 hypocotyls treated with 10 μM IAA, which would maximally stimulate endogenous ethylene production (Fig. 1), elongated normally under these conditions (Fig. 3).

To confirm the independence of VFN8 hypocotyl elongation from control by auxin-induced ethylene, ethylene synthesis was blocked by addition of 100 μM $CoCl_2$ or 1.2 μM AVG (24, 25). Although the inhibitors reduced ethylene synthesis in the presence of 10 μM IAA to, or below, the control (-IAA) rate, growth was not significantly affected (Table I). Similarly, silver thiosulfate, an inhibitor of ethylene action (21), also had no effect on auxin-induced elongation (Table I). Although the effectiveness of silver thiosulfate in inhibiting the action of ethylene in tomato hypocotyls is unknown, silver thiosulfate inhibited epinasty and promoted root growth in VFN8 plants (V Ursin, M Kelly, unpublished data). These effects are consistent with inhibition of ethylene action.

The responses of *dgt* and VFN8 hypocotyls to active and inactive IAA analogs or to inhibitors of auxin transport were also determined (Table II). Elongation of VFN8 hypocotyls in response to 10 μM 2,4-D, 2,4,5-T, pCPA, or αNAA was not significantly different from elongation in response to the same

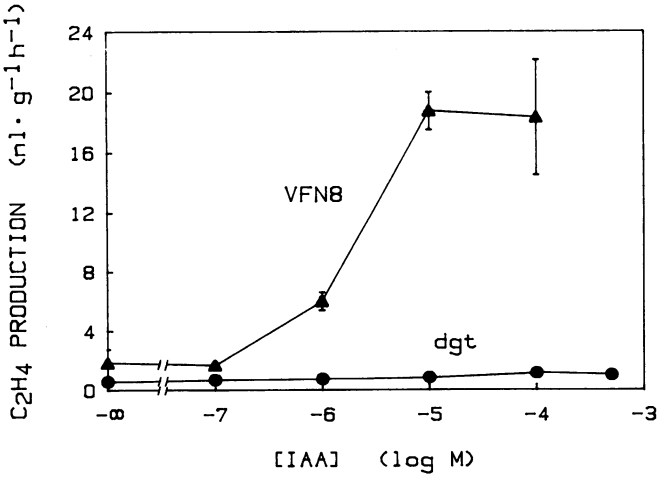

FIG. 1. Rate of ethylene production 2 to 5 h after treatment of etiolated VFN8 or *dgt* hypocotyls with the indicated concentration of IAA. Bars represent standard error of the mean where this value exceeds the width of the symbol.

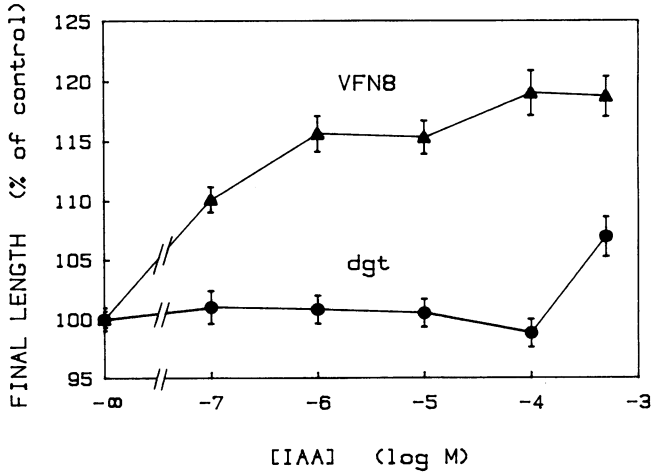

FIG. 2. Length of etiolated *dgt* or VFN8 hypocotyls after 10 h incubation with the indicated concentration of IAA. A representative experiment is presented. Bars represent standard error of the mean.

concentration of IAA. Growth in the presence of the inactive analogs oCPA or βNAA was essentially the same as the untreated control. *Dgt* hypocotyls failed to elongate significantly in response to any of these IAA analogs. Since the hypocotyls are bathed in the test solutions, auxin transport should not be a factor in these experiments. This was tested by including the auxin transport inhibitors TIBA or NPA in the assay. At concentrations which inhibited auxin transport in other species (7, 19), neither TIBA or NPA significantly affected IAA-induced elongation of VFN8 hypocotyls (Table II).

Failure of *dgt* hypocotyls to elongate markedly in response to the auxins tested raises the question of whether they are incapable of accelerated growth rates, invalidating hypocotyl elongation as an assay for auxin sensitivity. However, the fungal toxin fusicoccin dramatically promoted both elongation and ethylene production by VFN8 and *dgt* hypocotyls (Table III). The stimulation of ethylene synthesis rates by FC in both VFN8 and *dgt* hypocotyls was completely inhibited by AVG (Table III).

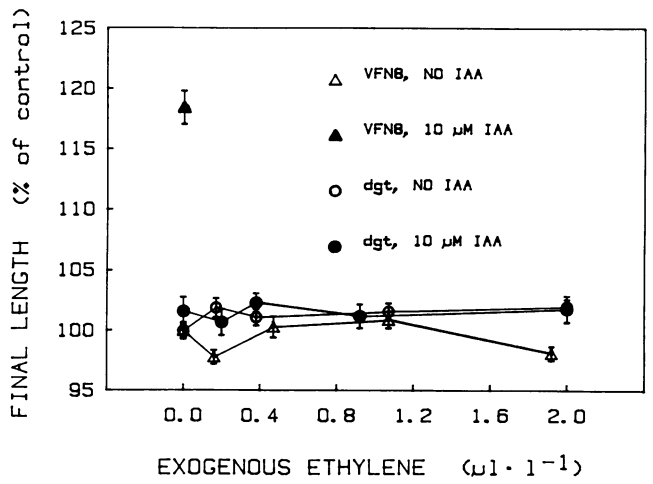

FIG. 3. Length of etiolated *dgt* or VFN8 hypocotyls after 10 h incubation with different concentrations of ethylene in the presence or absence of 10 μM IAA. Ethylene was not added to the IAA-treated VFN8 samples since they would be exposed to endogenously synthesized ethylene. Bars represent standard error of the mean.

DISCUSSION

These results confirm previous reports (3, 5, 27) that the *dgt* mutant has reduced sensitivity to IAA as an inducer of ethylene synthesis compared to its parent line, VFN8 (Fig. 1). The stimulation of ethylene synthesis by FC in both *dgt* and VFN8 tissues (Table III) is consistent with the conclusion that the mutant's ethylene-synthesizing capacity is normal except for reduced response to auxin (3, 5). As *dgt* tissues readily convert ACC to ethylene (3), it is apparently the induction of ACC synthase which is insensitive to IAA (24). FC-induced ethylene synthesis apparently occurs via the *S*-adenosylmethionine → ACC → ethylene pathway, as the rise in ethylene production was blocked by AVG, which inhibits the activity of ACC synthase (25).

A hypocotyl elongation assay was developed to test a second auxin response, presumably unrelated to the interaction of auxin and ethylene, to determine whether the insensitivity of the *dgt* mutant to auxin is restricted to the induction of ethylene synthesis. Etiolated hypocotyls of VFN8 elongated in response to IAA, with a concentration dependence similar to that observed in other growing tissues (Fig. 2) (2). Growth of normal tomato hypocotyls in these experiments (*e.g.* Fig. 2) was less than that reported in auxin assays with *Avena* coleoptiles (12, 13), but was similar in magnitude to that observed in light-grown tomato hypocotyls following treatment with brassinosteroids (20). Analogs of IAA reported to have growth promotory activity (pCPA, 2,4-D, 2,4,5-T, and αNAA) (17) stimulated elongation of normal hypocotyls, while the inactive isomers of pCPA and αNAA, oCPA and βNAA, respectively, were either ineffective or only marginally effective in inducing elongation (Table II). Although differential activity of the active analogs has been reported in a number of assays (17), the slight differences observed here were not statistically significant (Table II). The inability of 10 μM TIBA or NPA to prevent IAA-induced growth of VFN8 hypocotyls (Table II) indicates that polar auxin transport is not limiting to growth in this assay, in agreement with the conclusions of Katekar and Geissler (7) from experiments with pea stem sections. Two lines of evidence lead to the conclusion that auxin-induced elongation of normal tomato hypocotyls is not mediated by ethylene synthesized in response to auxin. First, growth of neither VFN8 nor *dgt* hypocotyls was promoted by up to 2 μl/L exogenous ethylene over a concentration range which should encompass the concentration of ethylene evolved following treatment with a growth-promotory level of auxin (0.22

Table I. *IAA-induced Elongation and Ethylene Evolution by VFN8 Hypocotyls as Influenced by Inhibitors of Ethylene Synthesis or Action*

The data for hypocotyl lengths are means ± SE of 15 to 20 segments; data for ethylene production are the means ± SE of 3 replicates with 15 segments each. A representative experiment is presented.

Treatment	Hypocotyl Length	Ethylene Production
	% of control	*nl g^{-1} h^{-1}*
Experiment 1		
Control	100.0 ± 0.8	1.16 ± 0.13
10 μM IAA	113.1 ± 1.4[a]	20.28 ± 4.19
100 μM $CoCl_2$	100.5 ± 0.6[b]	—[c]
100 μM $CoCl_2$, 10 μM IAA	111.4 ± 1.9[a]	1.27 ± 0.85
50 μM $Ag(S_2O_3)_2^{3-}$	101.6 ± 0.9[b]	—[c]
50 μM $Ag(S_2O_3)_2^{3-}$, 10 μM IAA	112.9 ± 1.3[a]	—[c]
Experiment 2		
Control	100.0 ± 1.0	0.74 ± 0.10
10 μM IAA	119.8 ± 1.6[a]	19.06 ± 1.90
1.25 μM AVG	99.6 ± 0.8[b]	—
1.25 μM AVG, 10 μM IAA	115.8 ± 1.9[a]	0.43 ± 0.05

[a] Values significantly different from untreated control ($P < 0.05$). [b] values not significantly different from untreated control. [c] Not measured.

Table II. *Elongation of VFN8 and dgt Hypocotyls in Response to IAA Analogs and Auxin Transport Inhibitors*

The data are the means ± SE of 15 to 20 segments. A representative experiment is presented.

Treatment	Final Length	
	dgt	VFN8
	% of control	
IAA, 10 μM	101.1 ± 0.9[a]	111.1 ± 1.5[b]
2,4-D, 10 μM	99.1 ± 1.3[a]	108.7 ± 0.8[b]
2,4,5-T, 10 μM	102.0 ± 1.0[a]	112.3 ± 1.6[b]
pCPA, 10 μM	100.3 ± 1.2[a]	108.9 ± 1.5[b]
oCPA, 10 μM	101.2 ± 1.4[a]	98.0 ± 0.6[a]
αNAA, 10 μM	100.2 ± 0.7[a]	112.2 ± 1.2[b]
βNAA, 10 μM	97.7 ± 0.7[a]	102.4 ± 0.8[a]
IAA, 10 μM	—[c]	116.8 ± 2.4[b]
IAA, 10 μM; NPA, 10 μM	—	114.3 ± 1.5[b]
IAA, 10 μM; TIBA, 10 μM	—	117.6 ± 2.5[b]

[a] Values not significantly different from untreated control. [b] Values significantly different from untreated control ($P < 0.05$). [c] Not tested.

Table III. *Elongation and Ethylene Evolution by dgt and VFN8 Hypocotyls in Response to 1.2 μM Fusicoccin*

Data for hypocotyl length are the means ± SE of 15 to 20 segments; data for ethylene synthesis are the means ± SE of 3 replicates with 15 segments each.

	Final Hypocotyl Length	Ethylene Snythesis Rate		
		Control	FC	FC, 1 μM AVG
	% of control	*nl g⁻¹ h⁻¹*		
VFN8	121.9 ± 1.1	1.54 ± 0.14	10.20 ± 1.78	0.82 ± 0.04
dgt	125.9 ± 1.5	1.13 ± 0.09	28.74 ± 5.23	0.77 ± 0.05

μl/L in the gas phase at the middle of the incubation). Second, inhibition of ethylene synthesis by Co^{2+} and AVG (25), or of ethylene action by Ag^{+} (21), did not influence the auxin-induced elongation of VFN8 hypocotyls (Table II). This assay is therefore specific for active auxins, is independent of polar auxin transport and ethylene synthesis, and satisfies the requirements for testing the auxin sensitivity of the *dgt* mutant in a system unrelated to auxin induction of ethylene synthesis.

Dgt hypocotyls did not elongate in response to IAA concentrations up to 100 μM (Fig. 2). The growth of *dgt* hypocotyls in response to 500 μM IAA (Fig. 2) or 1.2 μM FC (Table III) indicates that the mutant's insensitivity to auxin is not a consequence of an incapacity for rapid elongation. Apparent insensitivity to IAA could result from abnormal uptake, metabolism, or transport of IAA by *dgt* tissue. In preliminary experiments, no differences were detected between the genotypes in initial uptake of [^{14}C]2,4-D (M. Kelly, unpublished data). The inability of the active IAA analogs (Table II) to induce growth suggests that metabolic inactivation of IAA is not the cause of the failure of *dgt* hypocotyls to respond, as analogs such as 2,4-D are normally metabolized much more slowly than is IAA (1). As polar auxin transport appears not to be a factor in this assay (Table II), the failure of *dgt* hypocotyls to grow is apparently not due to an inability to transport auxin. Although auxin-induced ethylene synthesis is apparently not required for auxin stimulation of growth of normal tomato hypocotyls, endogenous (uninduced) ethylene synthesis rates were somewhat lower in etiolated *dgt* hypocotyls than in VFN8. It is possible that a threshold level of ethylene is required for sensitivity to auxin, and the endogenous ethylene in *dgt* may have been below that level. However, exogenous ethylene did not restore sensitivity of *dgt* to auxin in the hypocotyl elongation assay (Fig. 3). Furthermore, AVG reduced ethylene synthesis in VFN8 hypocotyls to very low rates, with no significant inhibition of auxin-induced growth. Thus, we conclude that the primary physiological lesion resulting from mutation of the *dgt* gene is a general insensitivity to auxin, and is not restricted to auxin-induced ethylene synthesis. We therefore propose that *dgt* is more properly termed the 'auxin-insensitive' than the 'ethylene-requiring' tomato mutant.

This conclusion is at odds with the observations that exogenous ethylene can phenotypically normalize *dgt* plants (5, 27, 28). However, application of inhibitors of ethylene synthesis or action did not induce the *dgt* phenotype in normal intact tomato plants (5; KJ Bradford, unpublished data). While there is little doubt that certain phenotypic characteristics of *dgt* can be made more normal by ethylene, it is perhaps only those processes which are limited by auxin-induced ethylene which respond to application of the gas. Thus, treatment of *dgt* with ethylene may not produce a truly 'normal' plant. Nonetheless, this could be a useful system for studying those processes which may be dependent upon the interaction of auxin and ethylene, such as geotropism (15, 23) or expansion of certain cell types (8, 14).

The biochemical consequence of the *dgt* mutation is not restricted to failure of auxin to induce the synthesis of ACC synthase. The reduced auxin effectiveness in two different responses, growth and ethylene production, as well as the multiple morphological abnormalities of *dgt* plants, point toward a defect associated with a primary site of auxin perception or action. The growth response of *dgt* hypocotyls to very high IAA levels (Fig. 2) suggests that it is the perception, rather than the response, which is at fault. The mutated *dgt* gene may code for a receptor molecule with reduced affinity for auxin, as was hypothesized previously (3). If so, the *dgt* mutant could provide a genetic tool for confirming the physiological activity of presumed auxin binding sites (16, 18). The data thus far, however, do not exclude the possibility that the lesion is in a primary response to IAA. The differential effectiveness of IAA and FC in stimulating growth and ethylene production of *dgt* hypocotyls provides an opportunity to test this hypothesis and to further discriminate between the mechanisms of action of the two growth regulators (10, 11). The *dgt* mutant is a unique genetic system in which to investigate the molecular mechanisms of auxin perception and action.

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