A Putative Role for the Tomato Genes *DUMPY* and *CURL-3* in Brassinosteroid Biosynthesis and Response[1]

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The *dumpy* (*dpy*) mutant of tomato (*Lycopersicon esculentum* Mill.) exhibits short stature, reduced axillary branching, and altered leaf morphology. Application of brassinolide and castasterone rescued the *dpy* phenotype, as did C-23-hydroxylated, 6-deoxo intermediates of brassinolide biosynthesis. The brassinolide precursors campesterol, campestanol, and 6-deoxocathasterone failed to rescue, suggesting that *dpy* may be affected in the conversion of 6-deoxocathasterone to 6-deoxoteasterone, similar to the Arabidopsis *constitutive photomorphogenesis and dwarfism* (*cpd*) mutant. Measurements of endogenous brassinosteroid levels by gas chromatography-mass spectrometry were consistent with this hypothesis. To examine brassinosteroid-regulated gene expression in *dpy*, we performed cDNA subtractive hybridization and isolated a novel xyloglucan endotransglycosylase that is regulated by brassinosteroid treatment. The *curl-3* (*cu-3*) mutant (*Lycopersicon pimpinellifolium* [Jusl.] Mill.) shows extreme dwarfism, altered leaf morphology, de-etiolation, and reduced fertility, all strikingly similar to the Arabidopsis mutant *brassinosteroid insensitive 1* (*bri1*). Primary root elongation of wild-type *L. pimpinellifolium* seedlings was strongly inhibited by brassinosteroid application, while *cu-3* mutant roots were able to elongate at the same brassinosteroid concentration. Moreover, *cu-3* mutants retained sensitivity to indole-3-acetic acid, cytokinins, gibberellin, and abscisic acid while showing hypersensitivity to 2,4-dichlorophenoxyacetic acid in the root elongation assay. The *cu-3* root response to hormones, coupled with its *bri1*-like phenotype, suggests that *cu-3* may also be brassinosteroid insensitive.

Brassinosteroids (BRs) are polyhydroxylated plant sterol derivatives with structural similarity to growth-regulating steroid hormones found in vertebrates and insects (Yokota, 1997). Nearly two decades of chemical and physiological analyses have resulted in an impressive catalog of responses elicited in plants treated exogenously with BRs, including enhanced stem elongation and vascular differentiation and an increased capacity to tolerate environmental stresses (for review, see Clouse and Sasse, 1998). The detailed characterization of the BR biosynthetic pathway (Fujioka and Sakurai, 1997), coupled with the discovery of BR-deficient and BR-insensitive mutants (Clouse and Feldmann, 1999), has provided convincing evidence that BRs are essential signal molecules controlling normal plant growth and development, and these steroids have now been widely accepted as a sixth class of plant hormones.

Several steps of the BR biosynthetic pathway in Arabidopsis have been characterized by feeding labeled intermediates followed by gas chromatography-mass spectrometry (GC-MS) analysis and/or by monitoring the effect of these compounds on the phenotype of BR-deficient mutants. For example, *constitutive photomorphogenesis and dwarfism* (*cpd*) is rescued to wild type by feeding teasterone and all other C-23 hydroxylated downstream intermediates, but not by cathasterone, which is lacking a C-23 hydroxyl (Szekeres et al., 1996). Moreover, the cloned *CPD* gene shows significant homology to mammalian cytochrome (Cyt) P-450 steroid hydroxylases, suggesting that CPD catalyzes the hydroxylation of cathasterone to teasterone (Fig. 1).

Similarly, the *dwarf4* (*dwf4*) gene is rescued by cathasterone, 6-deoxocathasterone, and all C-22 hydroxylated downstream intermediates, but not by campestanol or 6-oxo-campestanol. The cloned *DWF4* gene also shows homology to mammalian Cyt P-450 steroid hydroxylases, and thus DWF4 is thought to catalyze the conversion of campestanol to 6-deoxocathasterone and of 6-oxo-campestanol to cathasterone (Choe et al., 1998). Earlier steps in the pathway have also been identified. DET2 is a 5α-reductase that reduces (24*R*)-24-methylcholest-4-en-3-one to (24*R*)-24-methyl-5α-cholestan-3-one during the four-step conversion of campesterol to campestanol (Li et al., 1996; Fujioka et al., 1997; Noguchi et al., 1999). *DWF1* (Feldmann et al., 1989), allelic to *DIMINUTO1* (*DIM*1) (Takahashi et al., 1995) and *CABBAGE1* (*CBB*1) (Kauschmann et al., 1996), encodes an enzyme that catalyzes a step in the conversion of 24-methylenecholesterol to campesterol (Klahre et al., 1998; Choe et al., 1999a).

A BR-insensitive Arabidopsis mutant, termed *BR insensitive 1* (*bri1*), was identified by the ability of mutant plants
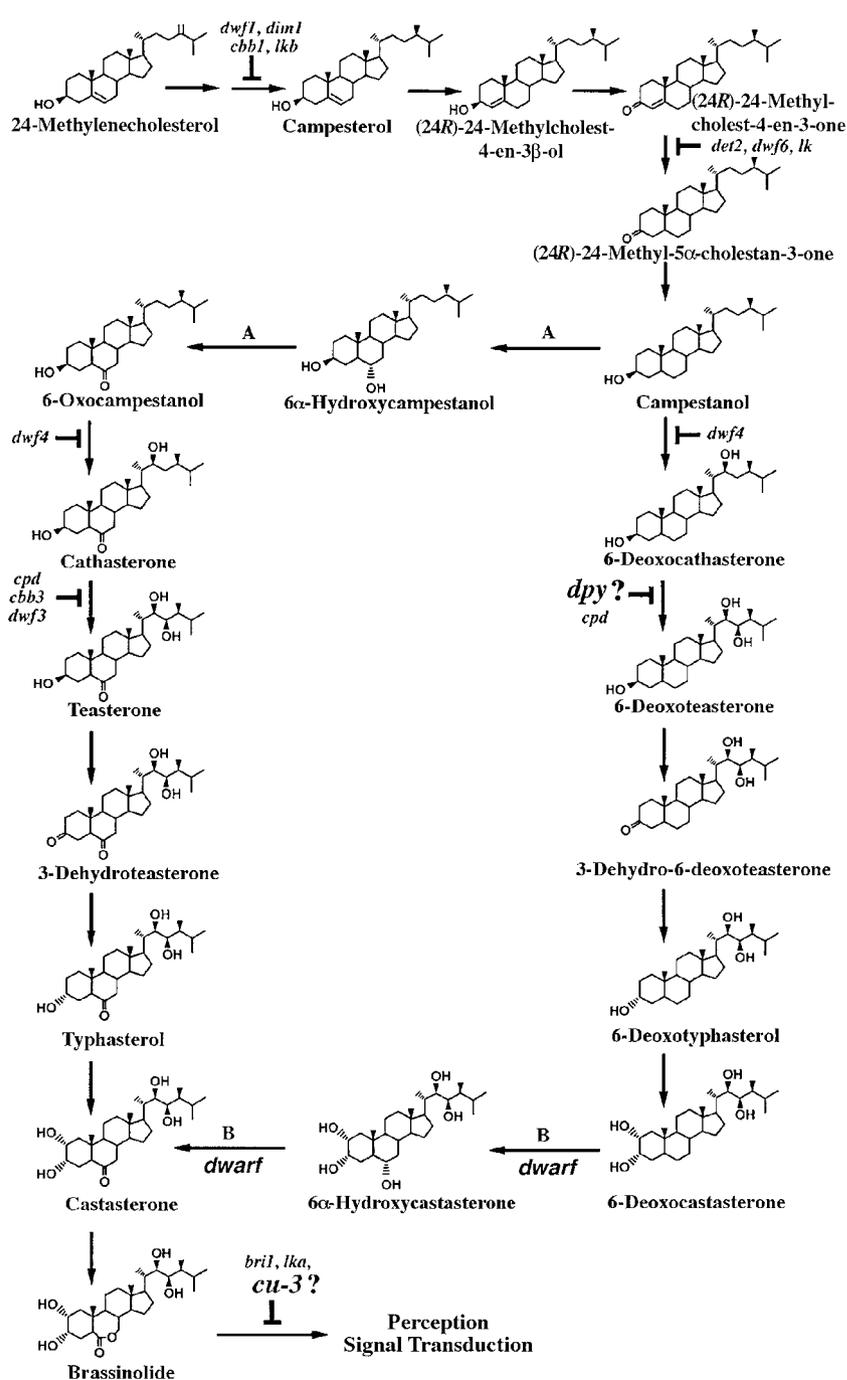
Figure 1. Brassinolide biosynthetic pathway displaying both early (A) and late (B) C-6 oxidation. Putative location of the deficient and insensitive Arabidopsis, pea (*lk, lka,* and *lkb*), and tomato (*dpy, dwarf,* and *cu-3*) mutants are indicated.

to elongate primary roots in the presence of BR concentrations inhibitory to wild-type root elongation (Clouse et al., 1993, 1996). Several alleles of *bri1* have been identified in independent screens (Kauschmann et al., 1996; Li and Chory, 1997; Clouse and Feldmann, 1999). The *BRI1* gene has recently been cloned and was found to encode a putative Leu-rich repeat receptor kinase most likely involved in an essential step of BR signal transduction (Li and Chory, 1997). The more severe BR mutants in Arabidopsis, including *bri1* and strong alleles of biosynthetic mutants such as *cpd*, show a characteristic phenotype that includes extreme dwarfism, dark green, shortened, and curled leaves, delayed senescence, and male sterility. Furthermore, Arabidopsis BR mutants are de-etiolated in the dark, with short hypocotyls, expanded cotyledons, and aberrant expression of light-regulated genes (Clouse and Feldmann, 1999).

Three pea dwarfs, *lk, lka,* and *lkb*, have also been shown to have altered BR response or biosynthesis. The *lka* dwarf does not respond to brassinolide treatment and thus is likely to function in BR signal transduction (Nomura et al., 1997). The dwarfism of *lk* and *lkb* are both rescued by BR treatment. The *lkb* mutant has severely reduced levels of

brassinolide, castasterone, 6-deoxocastasterone, campestanol, and campesterol, but has elevated levels of 24-methylenecholesterol, suggesting that LKB is the pea homolog of DWF1 in Arabidopsis (Nomura et al., 1997, 1999). Studies on the *lk* mutant have led to speculation that it serves the same function as DET2 in Arabidopsis (Yokota et al., 1997a). While the three pea mutants identified to date do show dwarfism, they do not exhibit the curled leaves and de-etiolation of the Arabidopsis BR mutants.

Tomato (*Lycopersicon esculentum*) is an important horticultural crop and an excellent model system for biochemical and genetic analysis of plant growth and development, particularly in those studies where the small size of Arabidopsis presents technical difficulties. Numerous naturally occurring mutants in tomato are available that are affected in hormone biosynthesis or response. For example, the *Never-ripe* mutant has been used in genetic analyses of fruit ripening (DellaPenna et al., 1989), and the *Never-ripe* gene is a homolog of *ETR*1, which encodes an ethylene receptor in Arabidopsis (Wilkinson et al., 1995). Other tomato mutants currently being utilized to gain an understanding of hormone action include *lateral suppressor* (Schumacher et al., 1995), *diageotropica*, an auxin-insensitive mutant (Kelly and Bradford, 1986); *flacca*, *notabilis*, and *sitiens*, abscisic acid (ABA)-deficient mutants (Taylor et al., 1988; Parry et al., 1992); and *gib1, gib2* and *gib3*, gibberellic acid (GA)-deficient mutants (Koornneef et al., 1990). To date, only the *dwarf* mutant of tomato, and its extreme allele d^x, have been identified as BR deficient based on plant phenotype, homology of the *DWARF* gene to *CPD*, feeding experiments with BR biosynthetic intermediates, and measurements of endogenous BR levels (Bishop et al., 1996, 1999).

The objective of the present study was to determine if any other of the known dwarf mutants of tomato result from lesions in genes encoding BR biosynthetic enzymes or signal transduction components. We assumed that mutants unable to synthesize or perceive BR would be associated with a dwarf phenotype, since BRs are known to be essential for cell elongation, and all Arabidopsis and pea BR mutants are of short stature. Therefore, application of exogenous BR was used as a screen to identify potential deficient mutants by rescue to wild-type phenotype. Furthermore, BRs are known to inhibit primary root elongation in tomato (Takatsuto et al., 1983; Roddick, 1994), and we adapted the Arabidopsis root elongation assay (Clouse et al., 1993) to tomato in an attempt to identify a BR-insensitive mutant . We present physiological, biochemical, and molecular evidence that the *L. esculentum* mutant *dumpy* (*dpy*) is likely to result from BR deficiency, while the *Lycopersicon pimpinellifolium* mutant *curl-3* (*cu-3*) is not rescued by BR treatment and is insensitive specifically to BRs in the root inhibition assay.

MATERIALS AND METHODS

Screening for BR-Deficient Mutants

Twenty-seven nonallelic, single gene dwarf mutants and their corresponding wild-type near isogenic lines were obtained from the Tomato Genetics Resource Center (Davis, CA). Of these, 26 were in the *Lycopersicon esculentum* background and one (*cu-3*) was in the *Lycopersicon pimpinellifolium* background. Seeds were sterilized by treatment with a 20% commercial bleach solution (1.05%, w/v, hypochlorite) for 40 min, rinsed in sterile water, and sown in a seedling flat with 4P potting medium (Fafard, Agawam, MA). Six seedlings from each accession were transplanted in 2.8-L plastic pots and grown in a greenhouse under natural light. Twenty days after seeding, three of the plants from each accession were sprayed until runoff with a 1.0 μM 24-epibrassinolide solution containing 0.01% (v/v) ethanol and 0.1% (v/v) Tween 20 (Fisher Scientific, Pittsburgh). The other three plants were treated with a solvent control. Treatments were applied once per week for 9 weeks. Since 24-epibrassinolide is more economical to synthesize than brassinolide, it was used exclusively in initial screening experiments.

The *dpy* mutant was selected for further evaluation based on its strong response to 24-epibrassinolide treatment. *dpy* seeds were sown in 4P potting medium and transplanted to 12.7-cm pots after 2 weeks of growth in the greenhouse under natural light. Twenty-one days after seeding, the plants were divided into 17 treatments of three pots each. Each set of three plants was then sprayed to runoff twice daily for an additional 21 d with either 1.0 μM campesterol, campestanol, 6-OH-campestanol, 6-oxo-campestanol, cathasterone, 6-deoxocathasterone, teasterone, 6-deoxoteasterone, 3-dehydroteasterone, 6-deoxo-3-dehydroteasterone, typhasterol, or 6-deoxotyphasterol; or 0.1 μM 6-deoxocastasterone, castasterone, or brassinolide. One set of three plants was treated as above with a solvent control and one set was left untreated. The entire experiment was repeated, with similar results. Figure 1 shows the structures of BRs and biosynthetic intermediates used in this experiment. These compounds were synthesized as previously described (Fujioka et al., 1997).

Quantitative Analysis of Endogenous BRs and Sterols

Thirty plants each of Alisa Craig (wild type) and *dpy* tomatoes were grown for 6 weeks in the greenhouse. The upper four nodes of each plant were excised and the material from each genotype was pooled, weighed, frozen in liquid nitrogen and lyophilized. Determination of endogenous BR levels in *dpy* and wild type was performed on extracts of pooled individuals of each genotype spiked with internal ^{2}H standards, which is widely accepted as the most accurate method of BR determination (Fujioka et al., 1997; Choe et al., 1998, 1999a, 1999b; Klahre et al., 1998; Nomura et al., 1999). Lyophilized plant materials (50 g fresh weight equivalent) from wild type or *dpy* mutants were extracted with 300 mL of MeOH-CHCl$_3$ (4:1) twice, and [^{2}H$_6$]brassinolide, [^{2}H$_6$]castasterone, [^{2}H$_6$]typhasterol, [^{2}H$_6$] teasterone, [^{2}H$_6$]6-deoxocastasterone, [^{2}H$_6$]6-deoxotyphasterol, and [^{2}H$_6$]6-deoxoteasterone (100 ng each) were added to the extract as internal standards. After evaporation of the solvent in vacuo, the extract was partitioned between CHCl$_3$ and water three times. The CHCl$_3$-soluble fraction was subjected to silica gel chromatography (Sep-

Pak Vac Silica, 35 mL, Waters, Milford, MA). The column was subsequently eluted with 100 mL of $CHCl_3$, 2% MeOH in $CHCl_3$, and 7% (v/v) MeOH in $CHCl_3$. Each 2% (v/v) MeOH and 7% (v/v) MeOH fraction was purified by Sephadex LH-20 column chromatography (column volume of 200 mL). The column was eluted with MeOH-$CHCl_3$ [4:1]. The effluents of elution volume/total column volume: 0.6 to 0.8 were collected as the BR fraction.

After purification on an ODS cartridge (Sep-Pak Plus C_{18}, Waters) with 20 mL of MeOH, eluates were subjected to ODS-HPLC (Pak ODS-4053-N; 10 × 50 mm + Pak ODS-5251-N; 20 × 250 mm, Senshu Scientific, Tokyo) at a flow rate of 8 mL min^{-1} with the solvents 90% (v/v) acetonitrile for the eluate derived from the 2% (v/v) MeOH fraction and 65% (v/v) acetonitrile for the eluate derived from the 7% (v/v) MeOH fraction. HPLC purification from the 7% (v/v) MeOH fraction yielded a brassinolide fraction (Rt 10–15 min), castasterone fraction (Rt 15–20 min), teasterone fraction (Rt 35–45 min), typhasterol fraction (Rt 45–55 min), and 6-deoxocastasterone fraction (Rt 65–80 min), and HPLC purification from the 2% (v/v) MeOH fraction yielded a 6-deoxoteasterone fraction (Rt 55–65 min) and 6-deoxotyphasterol fraction (Rt 65–90 min). Each fraction was analyzed by GC-MS after derivatization as previously described (Fujioka et al., 1997).

For sterols and 6-deoxocathasterone analysis, lyophilized plant materials (2 g fresh weight equivalent) from wild type and the *dpy* mutant were used. Plant materials were extracted with 50 mL of MeOH-$CHCl_3$ (4:1) twice, and [$^2H_7$]24-methylenecholesterol (4 μg), [$^2H_6$]campesterol (40 μg), [$^2H_6$]campestanol (1 μg), and [$^2H_6$]6-deoxocathasterone (6 ng) were added to the extract as internal standards. After evaporation of the solvent in vacuo, the extract was partitioned between $CHCl_3$ and water three times. The $CHCl_3$-soluble fraction was subjected to silica gel chromatography (Sep-Pak Vac Silica, 12 mL, Waters) and eluted with 20 mL of $CHCl_3$. The eluate was purified with an ODS cartridge as above. The eluent was subjected to ODS-HPLC (Pak ODS 4150-N; 10 × 150 mm, Senshu Scientific) at a flow rate of 2 mL min^{-1} with MeOH as the solvent. Fractions were collected every 0.5 min (between a Rt of 5 and 20 min). The main fractions of 6-deoxocathasterone and each sterol were as follows: 6-deoxocathasterone (Rt 6.5–7 min), 24-methylenecholesterol (Rt 13–13.5 min), campesterol (Rt 15.5–16 min), and campestanol (Rt 16.5–17 min). Each fraction was analyzed by GC-MS after derivatization as previously described (Fujioka et al., 1997).

Dark-Grown Seedling Studies

BR feeding experiments were performed in the dark by sterilizing *dpy* seeds as described above followed by placement on a straight line in 150- × 15-mm Petri plates containing 1% (w/v) agar, 2% (w/v) or 3% (w/v) Suc, and half-strength Murashige and Skoog medium at pH 5.7. Different BR biosynthetic intermediates were added to the sterile, cooled media just before pouring plates. All intermediates were used at 1.0 μM except for brassinolide, which was used at 0.2 or 0.5 μM. Plates were incubated vertically under continuous darkness at 25°C in a growth chamber. Seedlings were removed from the growth chamber after 9 or 11 d and photographed. Hypocotyl length of 10 seedlings for each treatment was measured to the nearest 1 mm.

For dark studies in liquid culture, seeds were sterilized as above and placed in 250-mL flasks containing 25 mL of half-strength Murashige and Skoog medium, pH 5.7, with 3% (w/v) Suc. Brassinolide and intermediates were added to the cooled medium at the same final concentrations used for the agar plate experiment. Flasks were incubated in a dark growth chamber at 24°C with constant shaking at 75 rpm. Hypocotyl length of 10 seedlings for each treatment was measured to the nearest 1 mm after 9 d of culture.

RNA Isolation and Analysis

Wild-type or *dpy* seeds were sterilized as described above and placed in 50 mL of half-strength Murashige and Skoog medium in Magenta jars. The jars were incubated in a growth chamber at 24°C under 50 μE m^{-2} s^{-1} light intensity (16 h of light/8 h of dark). After 21 d, plants were sprayed until runoff with a solvent control (0.01% [v/v] ethanol; 0.1% [v/v] Tween 20) or with 10^{-7} M brassinolide. The apices of the plants (the apical meristem and the immature first true leaf) were then harvested at 0, 1, 2, 4, 8, 12, and 24 h after treatment. Total RNA was isolated by grinding the tissue in liquid nitrogen, followed by homogenization in 5 mL of fresh extraction buffer (1% [w/v] triisopropylnaphthalene sulfonic acid; 6% [w/v] *p*-aminosalicylic acid; 100 mM Tris-HCl, pH 7.8; 50 mM EDTA; 100 mM NaCl; 1% [w/v] SDS; 78 μL/100 mL 2-mercaptoethanol) and 5 mL of phenol:chloroform:isoamyl alcohol (25:24:1). After centrifugation, the aqueous phase was re-extracted with phenol:chloroform:isoamyl alcohol and then subjected to three successive precipitations with ethanol/sodium acetate; 4.0 M LiCl; and ethanol/sodium acetate. The final pellet was resuspended in diethyl pyrocarbonate-treated water. Total RNA (25 μg) was analyzed on formaldehyde/MOPS gels, followed by alkaline transfer to Zeta-probe membranes (Bio-Rad, Richmond, CA). The full-length subtracted cDNA clone of *LeBR1* was used as a probe. Probe preparation and hybridization conditions were as previously described (Zurek and Clouse, 1994).

Subtractive Hybridization and DNA Sequence Analysis

Total RNA from the 1- and 4-h control or BR-treated *dpy* tissue was used for poly(A^+) RNA isolation (Promega, Madison, WI). The 1- and 4-h poly(A^+) samples of control or BR-treated tissue were pooled and used as driver (control) and tester (BR-treated) following the PCR-Select cDNA subtraction kit protocol exactly as described by the manufacturer (catalog no. K1804–1, CLONTECH, Palo Alto, CA). A portion of the subtracted cDNA thus generated was cloned into the pCR-Script vector using the pCR-Script cloning kit (Stratagene, La Jolla, CA). Differential screening of 96 independent colonies was performed as described in the PCR-Select instruction manual, and two clones representing distinct differentially expressed genes were identified. These were sequenced on both strands

using automated fluorescent DNA sequencing, and related sequences were obtained by BLASTX analysis (Altschul et al., 1990) at http://www.ncbi.nlm.nih.gov/BLAST/. The most closely related sequences were aligned by ClustalW 1.7 analysis at http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html. One clone, an xyloglucan endotransglycosylase (XET), is described in this paper, while the second clone, a putative soluble kinase, will be described elsewhere (C.V. Koka and S.D. Clouse, unpublished data).

Root Inhibition Assays

The *L. pimpinellifolium* mutant *cu-3* was investigated for sensitivity to BR using a screen adapted from Clouse et al. (1993). Seeds were sterilized as above and rinsed three times in a large volume of sterile water. Seeds were placed in a straight line in 15- × 100-mm Petri plates containing 1% (w/v) agar, 2% (w/v) Suc, pH 5.7, and one-half-strength Murashige and Skoog medium (Murashige and Skoog, 1962). 24-Epibrassinolide, at a final concentration of 0.5 μM, was added to the medium after autoclaving and cooling to <55°C. Plates were placed vertically in a growth chamber at 23°C with a 16-h light/8-h dark cycle at 50 μE m^{-2} s^{-1} intensity. Root length of all seedlings was measured to the nearest 0.1 cm after 9 d. Since *cu-3* has reduced fertility, segregating F$_2$ progeny from a mutant × wild-type cross were used for all experiments.

For 24-epibrassinolide dose response experiments, all treatments consisted of 10 replicate plates with 12 seeds each. Five wild-type seedlings and up to five *cu-3* mutants were randomly selected from each plate after 9 d and the root length was measured to the nearest 0.1 cm. The measurements within each plate were treated as subsamples and averaged among phenotypes. Seedlings with indistinguishable phenotypes were tagged and grown in vitro until they were scorable. Similarly, 10 replicate plates with 12 seeds each were incubated for 8 d for 2,4-dichloro-phenoxyacetic acid (2,4-D) and indole-3-acetic acid (IAA) dose response experiments. The number of replicates was increased to 12 plates with 12 seeds each for the other hormone sensitivity assays.

RESULTS

Phenotypes of *dpy* and *cu-3* and Their Response to BRs

Plants homozygous for the naturally occurring, spontaneous recessive mutation *dpy* have greatly condensed, dark-green rugose leaves that are downward curling and well spaced along the stem (Hernadez-Bravo, 1967). Moreover, *dpy* plants are shorter than the wild type and lack axillary branching. Figure 2A shows the phenotype of a *dpy* plant. Upon treatment with brassinolide, the *dpy* leaves lose their rugose characteristic and regain a wild-type appearance. When BRs are then withheld, new leaves on the mutant plants quickly revert to the *dpy* phenotype (data not shown). Nearly complete rescue of other wild-type characteristics, including normal plant stature and initiation and elongation of axillary branches, was also observed with

brassinolide treatment (Fig. 2A). As shown in Figure 2B, application of GA to *dpy* plants caused elongation of internodes, but had no effect on the characteristic leaf phenotype. The *cu-3* mutant was discovered among seedlings of *L. pimpinellifolium* (accession no. LA 1610) collected at Asia-El Pinon (Lima, Peru; Yu, 1982). It is a recessive single-gene mutant that shows many of the phenotypic characteristics of the Arabidopsis BR-insensitive mutant *bri1* (Clouse et al., 1996), including extreme dwarfism, dark-green curled leaves, delayed development, and reduced fertility (Fig. 2, C–F). In contrast to *dpy*, brassinolide treatment had no effect on the growth and development of the *cu-3* mutant (Fig. 2G). It was previously found that GA also could not rescue *cu-3* (Yu, 1982), and we confirmed this result (data not shown).

As seen in Figure 3A, both *dpy* and *cu-3* had short, thick hypocotyls in the dark, similar in size to the corresponding light-grown plants, but reached only 10% to 20% of the length of dark-grown wild-type hypocotyls. However, *dpy* seedlings retained a pronounced apical hook and closed cotyledons and thus were not truly de-etiolated to the degree observed in Arabidopsis BR mutants. Dark-grown *cu-3* mutants had a partial apical hook, but cotyledons were open to a greater extent than *dpy* seedlings grown under similar conditions. Application of 0.1 μM brassinolide to dark-grown *dpy* seedlings resulted in a nearly complete rescue to the wild-type phenotype (Fig. 3B).

The *dpy* Mutant Is BR Deficient

The rescue of *dpy* by exogenous BR suggests that the mutation lies in a gene encoding a BR biosynthetic enzyme. To determine the putative enzymatic activity disrupted in *dpy*, intermediates of the BR biosynthetic pathway (Fig. 1) were applied regularly for 21 d to a set of 51 *dpy* plants. Figure 4 shows that application of brassinolide and castasterone resulted in a nearly complete rescue of the mutant phenotype. The late C-6 oxidation pathway intermediates 6-deoxoteasterone, 6-deoxo-3-dehydroteasterone, 6-deoxo-typhasterol, and 6-deoxocastasterone also had a marked effect on *dpy* leaf development, although the overall plant height and branching were not as pronounced as with castasterone and brassinolide. However, intermediates upstream of 6-deoxoteasterone, including campesterol, campestanol, and 6-deoxocathasterone, had no effect on leaf phenotype or height beyond the variability normally seen in an untreated population of *dpy* mutants. The distinct difference in leaf morphology between *dpy* plants treated with 6-deoxoteasterone and later pathway intermediates compared with those treated with 6-deoxocathasterone and earlier pathway intermediates, suggests that conversion of 6-deoxocathasterone to 6-deoxoteasterone may be blocked in the *dpy* mutant. Early C-6 oxidation pathway intermediates also showed a distinct difference in leaf morphology between cathasterone and teasterone, although the effect of teasterone, 3-dehydroteasterone, and typhasterol on *dpy* were not nearly as dramatic as those of their late C-6 oxidation pathway counterparts (data not shown).

Rescue of dark-grown *dpy* seedlings by brassinolide and its biosynthetic intermediates was also consistent with a
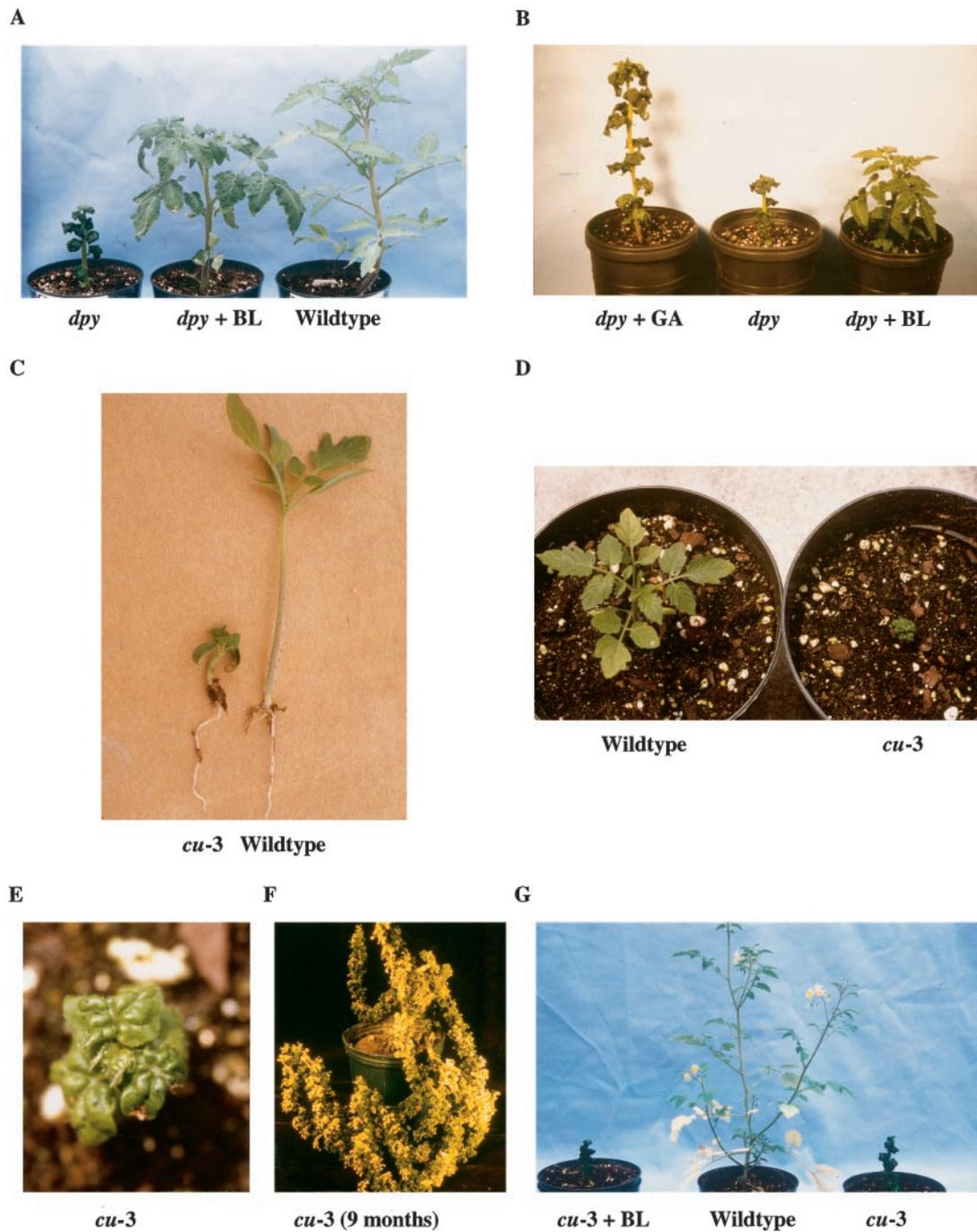
Figure 2. Characteristics of light-grown *cu-3* and *dpy* mutants. A, After 21 d of growth, *dpy* plants were treated with solvent control or 0.1 μM brassinolide (BL) twice daily for an additional 21 d, resulting in nearly complete rescue of the *dpy* phenotype to wild type. B, Ten-week-old *dpy* plants treated with solvent control (*dpy*), 1.0 μM 24-epibrassinolide, and 0.1 μM brassinolide twice per week (*dpy* + BL), or 1.0 μM GA$_3$ twice per week (*dpy* + GA). C, Fourteen-day-old light-grown *cu-3* and wild-type seedlings (untreated). D, The *cu-3* mutant displays extreme dwarfism (right) compared with a wild-type near-isogenic line grown under identical conditions (21 d, light-grown, untreated). E, Close-up of the *cu-3* mutant from D shows the curled-leaf phenotype. F, The *cu-3* mutant exhibits a delayed developmental program as displayed by this 9-month-old plant. G, After 21 d of growth, pairs of *cu-3* mutant plants were sprayed twice daily for an additional 21 d with a solvent control or 0.1 μM brassinolide. No rescue of the phenotype was observed.

site of action for the DPY gene product between 6-deoxocathasterone and 6-deoxoteasterone. Figure 5 shows that 9-d-old dark-grown *dpy* hypocotyls treated

with campesterol, campestanol, or 6-deoxocathasterone remained the same length as the solvent control, while hypocotyls treated with 6-deoxoteasterone and all down-
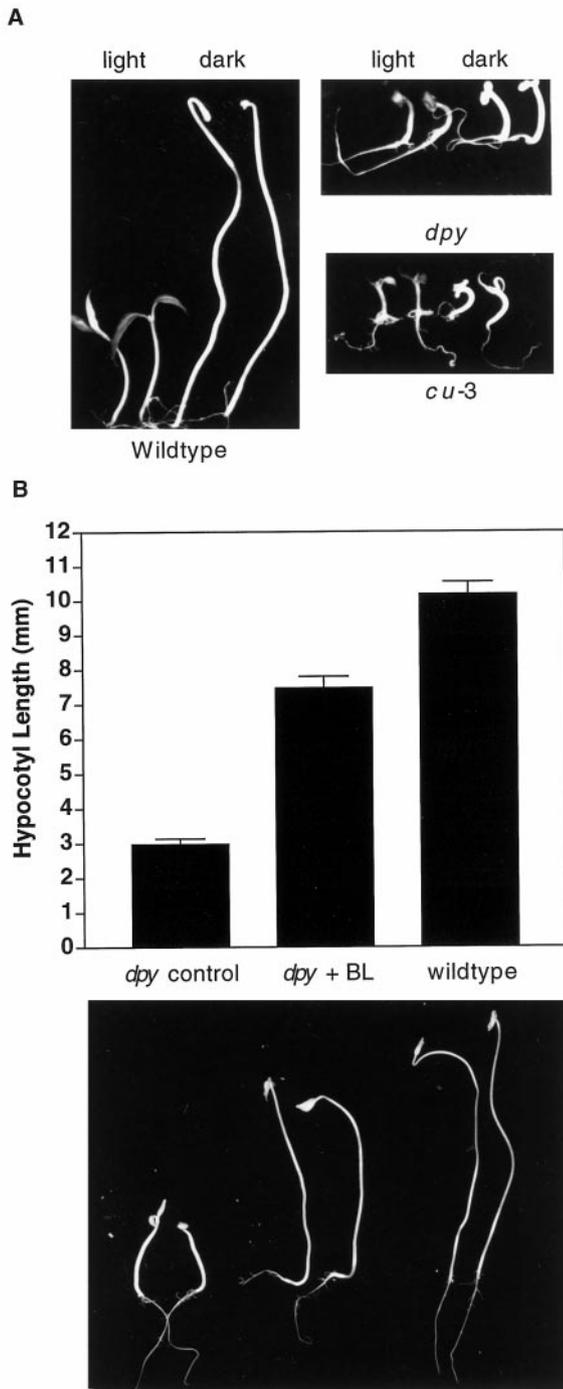
A

light dark   light dark

dpy

cu-3

Wildtype

B

Figure 3. Characteristics of dark-grown *cu-3* and *dpy* mutants. A, Phenotype of wild-type, *dpy*, and *cu-3* seedlings grown for 9 d in the light or dark in agar medium containing 2% (w/v) Suc, half-strength Murashige and Skoog salts, and 1% (w/v) agar. B, Seedlings grown in the dark for 11 d on the surface of the medium in vertically oriented Petri plates showed nearly complete rescue to wild-type length when 0.2 μM brassinolide (BL) was included in the medium. Hypocotyl length of 10 seedlings for each treatment was measured to the nearest 1 mm ± SE. The photograph below the bar graph shows representative seedlings for each treatment.

stream intermediates showed significant elongation. Similar results were obtained on agar plates or in shaking liquid medium, but the mean hypocotyl length was greater in the liquid culture.

To verify that *dpy* was indeed BR deficient, endogenous BR levels were measured by GC-MS with internal ^{2}H standards in 42-d-old *dpy* and wild-type plants grown under identical conditions. As shown in Table I, both *dpy* and wild-type plants had similar levels of the BR precursors 24-methylenecholesterol, campesterol, and campestanol. However, *dpy* plants contained twice the level of 6-deoxocathasterone as the wild type and less than half the amount of 6-deoxoteasterone. Moreover, *dpy* plants had a 25-fold reduction in 6-deoxocastasterone levels, and castasterone was below the limit of detection in the mutant. Thus, *dpy* is clearly BR deficient and the biochemical data are consistent with the feeding experiments suggesting that *dpy* mutants have reduced conversion of 6-deoxocathasterone to 6-deoxoteasterone.

dpy Shows Reduced Expression of a BR-Regulated Gene

If *dpy* is indeed BR deficient, we would expect reduced expression of BR-regulated genes in mutant versus wild-type plants grown under similar conditions. Moreover, since *dpy* retains BR sensitivity, an increase in transcript levels for these genes should be observed upon treatment of the mutant with BR. The reduced level of endogenous BRs in the *dpy* mutant provides an excellent control tissue for such studies of BR-regulated gene expression. We used subtractive hybridization to clone genes that showed increased mRNA transcript levels when *dpy* was treated with BR.

The first gene isolated, *LeBR1* (accession no. AF205069), showed much higher transcript levels in BR-treated tissue than in control tissue at all time points examined, from 2 to 24 h after BR application (Fig. 6). Furthermore, in untreated tissue, expression of *LeBR1* was reduced in the *dpy* mutant compared with wild type. Moreover, the translated LeBR1 putative protein showed extensive sequence identity (79% identical, 91% similar) to BRU1, which we previously showed was encoded by a BR-regulated XET gene that is highly expressed in elongating soybean stems (Zurek and Clouse, 1994). BLAST analysis showed that LeBR1 also shared significant sequence identity with numerous other XETs in the database including 75% identity/86% similarity with Arabidopsis XTR-7 (Xu et al., 1996); 68% identity/80% similarity with tXET-B1 and 71% identity/83% similarity with tXET-B2 from tomato (Arrowsmith and de Silva, 1995); 66% identity/78% similarity with TCH4 from Arabidopsis (Xu et al., 1995); and 53% identity/72% similarity with LeEXT from tomato (Okazawa et al., 1993). Figure 7 shows the extent of sequence conservation between LeBR1 and other XETs, including the invariant DEIDEFLG, which is thought to contain the active site of the enzyme (Borriss et al., 1990).

Root Inhibition Assays and Hormone Sensitivity

To screen for BR insensitivity among the dwarf mutants, the vertical Petri plate root elongation assay previously
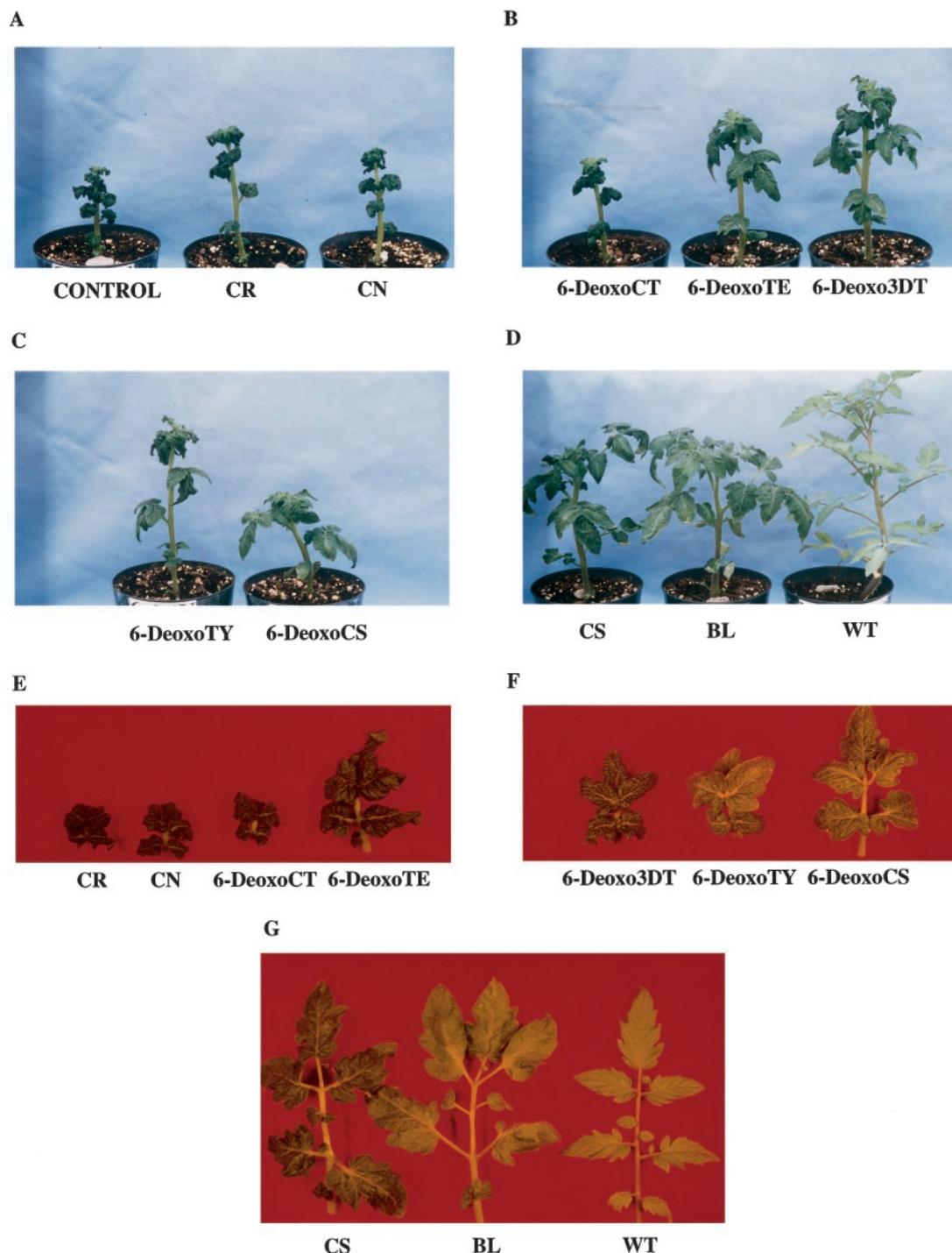
Figure 4. Feeding experiments with light-grown *dpy* plants. A to D, After 21 d of growth in a greenhouse, intermediates of the brassinolide biosynthetic pathway were applied to *dpy* plants twice daily for an additional period of 21 d. Campesterol through 6-deoxotyphasterol were applied at 1.0 μM and 6-deoxocastasterone, castasterone, and brassinolide were applied at 0.1 μM. Control and wild-type plants were sprayed with solvent only. E to G, Close-up of the third true leaves from plants in A to D. CR, Campesterol; CN, campestanol; 6-DeoxoCT, 6-deoxocathasterone; 6-DeoxoTE, 6-deoxoteasterone; 6-Deoxo3DT, 3-dehydro-6-deoxoteasterone; 6-DeoxoTY, 6-deoxotyphasterol; CS, castasterone; BL, brassinolide; WT, wild type.

used by Clouse et al. (1993, 1996) to identify BR-insensitive mutants in Arabidopsis was adapted for use in tomato. Wild-type Arabidopsis root elongation is severely inhibited by medium containing 0.5 μM 24-epibrassinolide (Clouse et al., 1993). Wild-type *L. esculentum*, *L. pimpinellifolium*, and the *dpy* mutant also demonstrated this inhibition of root elongation. However, roots of *cu-3* mutants were less sensitive, attaining a length comparable to
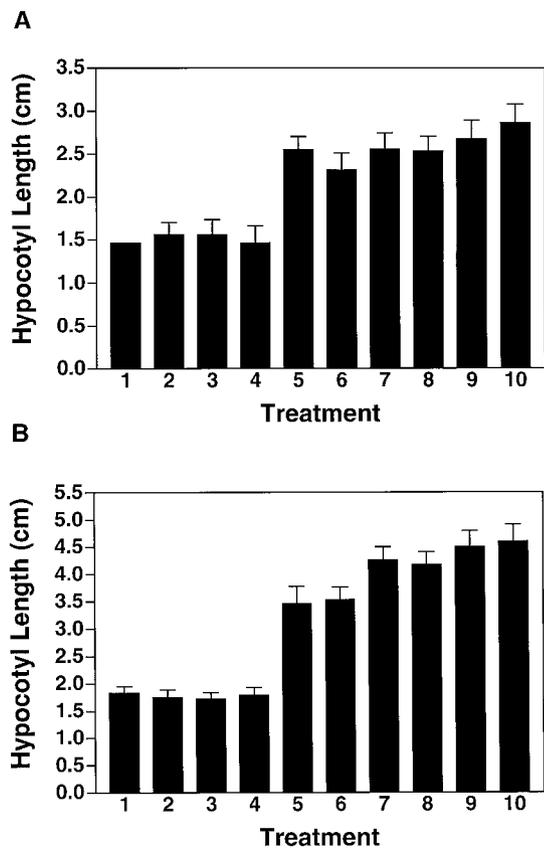
A

B

Figure 5. Feeding experiments with dark-grown *dpy* plants. A, *dpy* seeds were sterilized and plated on vertical Petri plates containing various brassinolide biosynthetic intermediates in 1% (w/v) agar, 3% (w/v) Suc, and half-strength Murashige and Skoog medium, pH 5.7. Seedlings were grown in complete darkness for 9 d and hypocotyl length of 10 seedlings for each treatment was measured to the nearest 1 mm ± SE. 1, Solvent control; 2, campesterol; 3, campestanol; 4, 6-deoxocathasterone; 5, 6-deoxoteasterone; 6, 6-deoxo-3-dehydroteasterone; 7, 6-deoxotyphasterol; 8, 6-deoxocastasterone; 9, castasterone; and 10, brassinolide. Intermediates 2 to 9 were applied at 1.0 μM and brassinolide was applied at 0.5 μM. B, Hypocotyl length of 10 seedlings ± SE grown in shaking liquid culture in the dark for 9 d. Biosynthetic intermediate concentrations are as described in A.

solvent-treated controls (Fig. 8). Interestingly, the *cu-3* mutant exhibited a phenotype very similar to the Arabidopsis *bri1* mutant, including extreme dwarfism, dark-green, curled leaves, delayed development, and reduced fertility.

To further characterize the BR insensitivity of the *cu-3* mutant, inhibition of root elongation was tested over a wide range of 24-epibrassinolide concentrations (10^{-12} to 10^{-6} M). As shown in Figure 9A, wild-type root elongation was inhibited in a dose-dependent manner as concentrations of 24-epibrassinolide increased, with a marked decrease in root length at 10^{-8} to 10^{-6} M 24-epibrassinolide. In contrast, the *cu-3* mutant maintained a generally consistent root length independent of 24-epibrassinolide treatments at lower concentrations ($<10^{-8}$ M), with only a slight inhibition of root elongation at higher concentrations.

The *cu-3* mutant was also tested with other plant hormones to determine if the insensitivity was BR specific. For

Table I. *Endogenous levels of sterols and BRs in dpy and wild-type tomato plants*

Compound	Wild Type	*dpy*	Wild Type/*dpy*
Sterols (ng/g fresh wt)			
24-Methylenecholesterol	351	393	0.89
Campesterol	2,600	2,500	1.04
Campestanol	33	29	1.14
BRs (pg/g fresh wt)			
6-Deoxocathasterone	1,200	2,600	0.46
6-Deoxoteasterone	65	27	2.41
6-Deoxocastasterone	907	36	25.19
Castasterone	66	n.d.[a]	
[a] n.d., None detected.			

all hormones tested, *cu-3* showed sensitivity equal to or greater than that of the wild type. Dose response curves for IAA (Fig. 9B) and 2,4-D (Fig. 9C) showed that *cu-3* was hypersensitive to 2,4-D over a wide range of concentrations, reminiscent of the Arabidopsis mutant *bri1* (Clouse et al., 1996), but that *cu-3* and the wild type responded to IAA equally; except at very low 24-epibrassinolide concentrations, where *cu-3* again showed some hypersensitivity. Cytokinins (kinetin and benzylaminopurine at 10 μM), ABA (0.5 μM). and GA$_3$ (1.0 mM) inhibited root elongation of wild-type and *cu-3* seedlings equally (Fig. 10).

DISCUSSION

We examined a collection of naturally occurring tomato dwarf mutants for possible BR deficiency or insensitivity. The dramatic change in *dpy* leaf morphology following exogenous 24-epibrassinolide treatment and the nearly complete rescue of the wild-type phenotype upon exogenous brassinolide application suggests that the *dpy* mutant
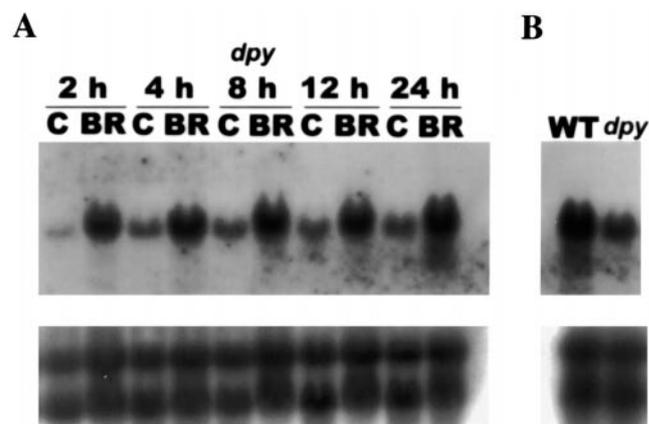

Figure 6. RNA-blot analysis of *LeBR1*, a BR-regulated tomato XET. Total RNA (25 μg) was analyzed by northern blots using ^{32}P-labeled *LeBR1* cDNA as the probe. Below is shown the stripped blot rehybridized with a rRNA probe as a loading control. A, Solvent control (C) or 10^{-7} M brassinolide (BR) were applied to *dpy* plants grown in the light for 21 d. RNA was extracted from apical meristems and the first true leaves harvested at the times indicated after solvent or brassinolide treatment. B, In a separate experiment, RNA was isolated from apical meristems and the first true leaves of untreated wild-type (WT) or *dpy* plants grown in the light for 21 d.

Figure 7. Multiple sequence alignment of LeBR1 with other XETs. ClustalW version 1.7 was used to align LeBR1 with other known XETs from soybean (BRU1), Arabidopsis (XTR-7, TCH4), and tomato (tXET-B1, tXET-B2, LeEXT). Amino acids identical to those in LeBR1 are shaded in black. The sequence DEIDFEFLG, presumed to be the active site of XETs, is indicated by asterisks.


```
1 Le-BR1  TYGGNFYQEFDFTWGGNRAKIFNGGQLLSLSLDKVSGSGFQSKKEHLFGRIDMQIKLVAG
2 BRU1     TCAGSFYQDFDLTWGGDRAKIFNGGQLLSLSLDKVSGSGFKSKKEYLFGRIDMQLKLVAG
3 XTR-7    AYASNFFDEFDLTWGDHRCKIFNGGNMLSLSLDQVSGSGFKSKKEYLFGRIDMQLKLVAG
4 tXET-B1  VWADNFYQDATVTFGDQRAQIQDGGRLLALSLDKISGSGFOSKNEYLFGRFDMQLKLVPG
5 tXET-B2  VSADNFYQDAAVTFGDQRAQIQDGGRLLTLSLDKISGSGFQSKNEYLFGRFDMQLKLVPG
6 TCH4     SVSANFQRDVEITWGDGRGQIKNNGELLTLSLDKSGSGFOSKNFYLFGKVSMQMKLVPG
7 LeEXT    PVDVFFWKNYEPSWASHHIKFLNGGTTTDLILDRSSGAGFQSKKSYLFGHFSMKMRLVGG
                                               *********
1 Le-BR1  NSAGTVTTYYLSSQGPTHDEIDFEFLGNVTGEPYILHTNIYAQGKGNKEQQFYLWFDPTK
2 BRU1     NSAGTVTAYYLSSQGPTHDEIDFEFLGNLSGDPYILHTNIFTQGKGNREQQFYLWFDPTR
3 XTR-7    NSAGTVTAYYLSSQGATHDEIDFEFLGNETGKPYVLHTNVFAQGKGDREQQFYLWFDPTK
4 tXET-B1  NSAGTVTTFYLSSQAGHDEIDFEFLGNSSGQPYTVHTNVYSQGKGNKEQQFRLWFDPTS
5 tXET-B2  NSAGTVTTFYLSSQGAGHDEIDFEFLGNSSCLPYTVHTNVYSQGKGNKEQQFRLWFDPTS
6 TCH4     NSAGTVTTLYLKSPGTTWDEIDFEFLGNSSGEPYLHTNVYTQGKGDKEQQFKLWFDPTA
7 LeEXT    DSAGVVTAFYLSSNNAEHDEIDFEFLGNRTGQPYIICTNVFTGGKGNREQRIYLWFDPTK
                                               *********
1 Le-BR1  NFHTYSIIWKPQHIIFLVDNTPIRVYKNAESVGVPRPR
2 BRU1     NFHTYSIIWKPQHIIFLVDNTPIRVFKNAEPLGVFFK
3 XTR-7    NFHTYSIVWRPQHIIFLVDNLPIRVFNNAEKLGVFFK
4 tXET-B1  PFHTYSIVWNSQRIIFLVDNIPIRVFNNHEKLGVAFFK
5 tXET-B2  SFHTYSIVWNSQRIIFLVDNIPIRVFNNHEALGVAYFK
6 TCH4     NFHTYTILWNPQRIIFTVDGTPIREFKNMESLGTLFFK
7 LeEXT    GYHSYSVLWNTYLIVIFVLDVPIRAFKNSKDLGVKFFF
```

is BR deficient and that the *DPY* gene product is involved in BR biosynthesis. The lack of 100% rescue to wild-type height by exogenous brassinolide treatment was also seen in another BR-deficient tomato mutant, *dwarf* (and its allele *d^x*), which was attributed to poor BR transport in tomato (Bishop et al., 1996, 1999). We verified that *dpy* is BR deficient with measurements of endogenous BR levels by GC-MS in mutant versus wild-type plants of the same age grown under identical conditions. Castasterone was below the detectable level and 6-deoxocastasterone was reduced 25-fold in *dpy* plants compared with the wild type.

The feeding of intermediates in the BR biosynthetic pathway to BR mutants has been widely used to locate the position in the pathway affected by the mutation. When we applied intermediates of both the early and late C-6 oxidation pathway to *dpy* plants in either the dark or the light, the most dramatic difference in phenotype occurred between 6-deoxocathasterone and 6-deoxoteasterone. It is well known that in bioassays with wild-type plants, the biological activity of BRs increases with position along the biosynthetic pathway (Fujioka et al., 1995). It has also been shown routinely in rescue experiments of BR-deficient mutants in pea and Arabidopsis that precursors earlier in the pathway become gradually less efficient in rescuing the mutant than castasterone or brassinolide, even though these intermediates are downstream of the mutation. For example, Choe et al. found that 10^{-6} M 6-deoxocathasterone and 6-deoxoteasterone had very similar effects on pedicel elongation of *dwf4* (which is blocked in the conversion of campestanol to 6-deoxocathasterone), which were equivalent to only 10^{-7} M brassinolide (Choe et al., 1998).

Similar results were obtained with mutants lying earlier in the pathway, such as *dwf7/ste* 1 (Choe et al., 1999b), *det*2 (Fujioka et al., 1997), and *dwf1/dim*1 (Klahre et al., 1998; Choe et al., 1999a) in Arabidopsis; and *lkb* (Nomura et al., 1999) in pea. In each case, 6-deoxocathasterone and/or 6-deoxoteasterone showed partial rescue compared with brassinolide, but clearly had biological activity and af-

fected the phenotype compared with the mutant control. Thus, in the case of *dpy*, the fact that 6-deoxocathasterone treatment is indistinguishable from the mutant control, while 6-deoxoteasterone treatment results in larger plants with more normal leaves in the light and increased hypocotyl length in the dark, strongly suggests that *dpy* is blocked in the conversion of 6-deoxocathasterone to 6-deoxoteasterone. This is supported by an increase of endogenous 6-deoxocathasterone levels and a decrease in 6-deoxoteasterone levels in the mutant compared with the wild type.

Two alternative pathways for brassinolide biosynthesis, early and late C-6 oxidation, (Fig. 1), were identified by feeding studies in cultured cells of *Catharanthus roseus* (Choi et al., 1996, 1997). The co-occurrence of 6-oxo and 6-deoxo intermediates in Arabidopsis suggested that both
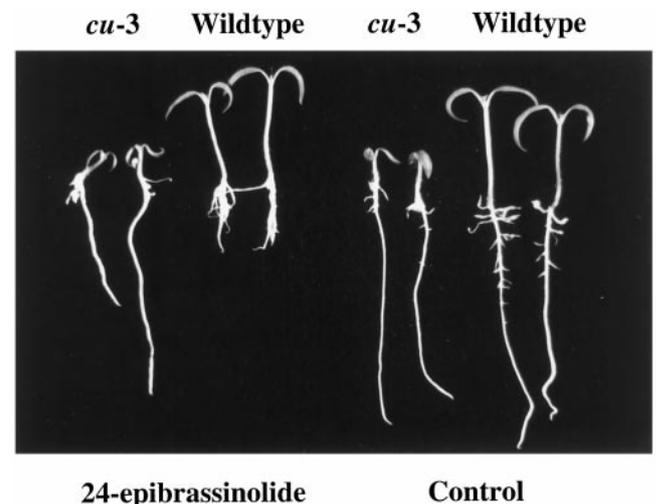

Figure 8. Effect of BR on root elongation in *cu-3* mutants. Primary root elongation of wild-type seedlings was inhibited when grown in medium containing 0.5 μM 24-epibrassinolide, but primary roots of *cu-3* mutants reached a length comparable to untreated controls.
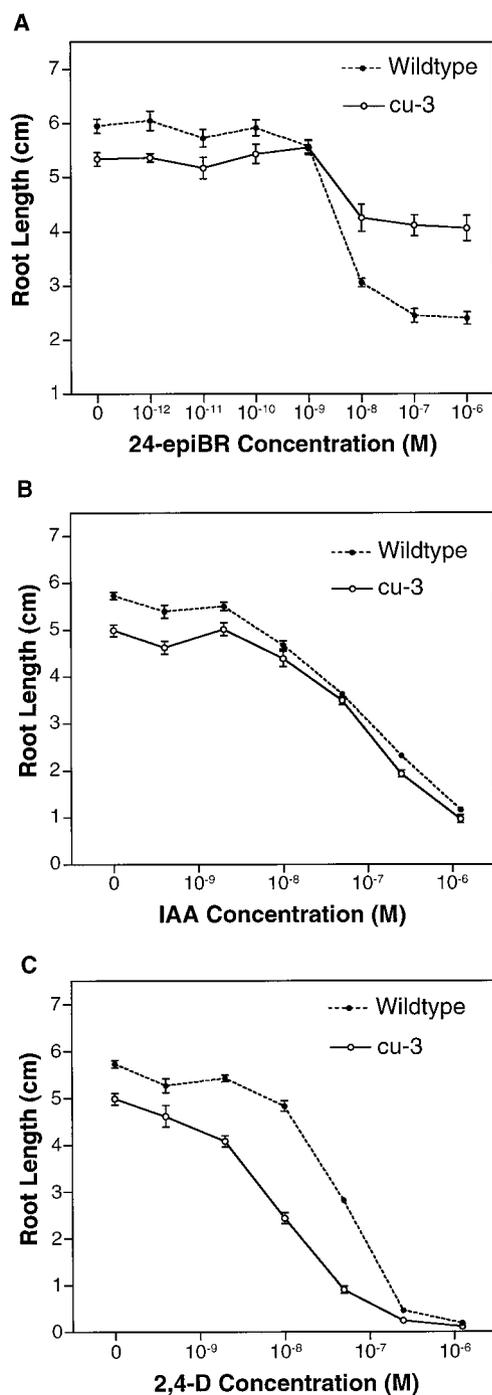
A

B

C

Figure 9. Dose-response curve of *cu-3* root elongation on media with BR and auxins. Wild-type (●) or *cu-3* (○) seedlings were grown in the light for 9 d on vertically oriented Petri plates containing the indicated hormone concentrations. Root length was measured to the nearest 0.1 cm. Data points are the means ± SE from a minimum of 10 replications.

the early C-6 oxidation pathway are more active in the dark (Fujioka et al., 1997; Choe et al., 1998).

In contrast to Arabidopsis, recent work in tomato (Yokota et al., 1997b; Bishop et al., 1999) showed that late C-6 oxidation intermediates were present, but early C-6 oxidation intermediates such as teasterone and typhasterol were not detected. In the present experiments, we were also able to detect late C-6 intermediates (Table I) but teasterone and typhasterol were again not detected (data not shown). Moreover, our observations on the rescue of the *dpy* mutant by BR biosynthetic intermediates are consistent with a predominant role for the late C-6 oxidation pathway in tomato. Significant rescue of the *dpy* phenotype to wild type was observed with 6-deoxoteasterone, 6-deoxo-3-dehydroteasterone, 6-deoxotyphasterol, and 6-deoxocastasterone in the light. A similar rescue did not occur with the early C-6 oxidation intermediates teasterone, 3-dehydroteasterone, and typhasterol, although a slight change in leaf morphology did occur when these compounds were supplied to the *dpy* mutant. Unlike the results in Arabidopsis (Fujioka et al., 1997; Choe et al., 1998), we also found that 6-deoxo intermediates were much more active than 6-oxo intermediates in the dark. It is highly unlikely that these differences in activity are based on differential uptake or stability of 6-oxo versus 6-deoxo compounds, since the 6-oxo compound brassinolide is readily taken up by tomato.

Based on feeding experiments and sequence analysis, the *CPD* gene of Arabidopsis has been proposed to encode a C-23 steroid hydroxylase responsible for the conversion of cathasterone to teasterone (Szekeres et al., 1996). CPD must also serve to hydroxylate 6-deoxocathasterone to 6-deoxoteasterone, since the involvement of a different enzyme in the late C-6 oxidation pathway would allow synthesis of brassinolide in the single gene *cpd* mutant, and thus a dwarf phenotype would not be observed. Our current results suggest that *DPY* may be the tomato homolog
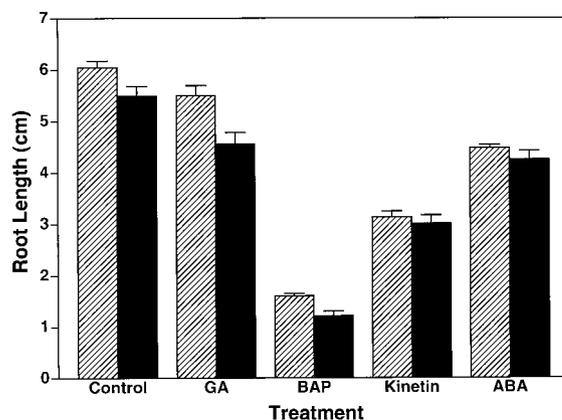

Figure 10. Root elongation of *cu-3* seedlings in the presence of various hormones. Wild-type (hatched bars) or *cu-3* (black bars) seedlings were grown in the light for 8 d on vertically oriented Petri plates containing GA_3 (1 mM), benzylaminopurine (10 μM), kinetin (10 μM), or ABA (0.5 μM). Root length was measured to the nearest 0.1 cm. Data points are the means ± SE from a minimum of 12 replications.

early and late C-6 oxidation pathways were operational in this species as well (Fujioka et al., 1996). Based on feeding experiments in Arabidopsis, it has been proposed that 6-deoxo intermediates of the late C-6 oxidation pathway are more active in the light, while 6-oxo intermediates of

of *CPD*, but verification must await the cloning of the *DPY* gene. It is also possible that *DPY* encodes an essential regulatory protein required for the 6-deoxocathasterone to 6-deoxoteasterone conversion, or that this conversion may in fact be a multistep process, with *DPY* encoding an essential, but distinct enzyme from the CPD homolog.

To examine altered BR regulation of gene expression in the *dpy* mutant, we performed subtractive hybridization of cDNAs derived from RNAs of *dpy* plants treated with or without BR, and isolated a novel XET whose expression was enhanced by BR at all times tested, from 2 to 24 h. XETs generally occur as differentially regulated multi-gene families whose expression is often associated with expanding tissue (Clouse, 1997). In one model of wall extension, expansins are proposed to primarily affect wall relaxation, while glucanases and XETs affect the extent of expansin activity by altering the viscosity of the hemicellulose matrix (Cosgrove, 1997). XETs may also function to incorporate new xyloglucan into the growing wall. BRs alter the biophysical properties of plant cell walls (Wang et al., 1993; Tominaga et al., 1994; Zurek et al., 1994) and also increase the abundance of mRNA transcripts for wall-modifying proteins such as XETs in soybean, Arabidopsis, and tomato (Zurek and Clouse, 1994; Xu et al., 1995; Catala et al., 1997).

It is interesting that *LeBR1* is more closely related to soybean *BRU1* than to other members of the tomato XET family. In elongating soybean epicotyls, BR application results in increased plastic extensibility of the walls within 2 h, with a concomitant increase in *BRU1* mRNA levels (Zurek and Clouse, 1994). The *BRU1* gene is regulated specifically by BRs during the early stages of elongation, and enzyme assays with recombinant protein show that BRU1 is indeed a functional XET (Oh et al., 1998). Moreover, the mechanism of BR regulation of *BRU1* has been shown to be posttranscriptional. Other BR-regulated genes, such as *TCH4* and *LeEXT*, are regulated by both BR and auxin, and *TCH4* has been shown to be transcriptionally regulated by BR. Thus, it will be informative to examine the specificity and mechanism of regulation of *LeBR1* to determine if, like *BRU1*, it is regulated specifically by BRs at the posttranscriptional level.

The inhibitory effect of BR on primary root elongation in Arabidopsis was critical in the identification of the *bri1* mutant (Clouse et al., 1996). Studies with various BRs differing in side chain structure have been conducted with excised roots (Roddick, 1994) and seedlings (Takatsuto et al., 1983) of tomato. In these assays the order of potency was brassinolide > 24-epibrassinolide > 22,23,24-trisepibrassinolide > 28-homo-brassinolide. Since tomato displays inhibition of root growth at high BR concentrations, it was assumed that either brassinolide or 24-epibrassinolide could potentially be used to identify insensitive tomato mutants based on the ability of roots to elongate in the presence of normally inhibitory concentrations of BR. We used the root elongation assay to demonstrate that the *cu-3* mutant maintained insensitivity over a wide range of 24-epibrassinolide concentrations. Even though some inhibition of root elongation was observed at higher concentrations of 24-epibrassinolide (10^{-8} to 10^{-6} M), it was proportionally much less than wild-type seed-

lings at the same 24-epibrassinolide concentrations. The *cu-3* mutant retained its sensitivity to GA, cytokinins, ABA, and IAA, and displayed a hypersensitive response to 2,4-D over a range of concentrations. These results are strikingly similar to those found with the *bri1* mutant of Arabidopsis (Clouse et al., 1996). However, *cu-3* did not exhibit the hypersensitivity to ABA shown by *bri1*, at least at the single concentration tested. The similarity between *cu-3* and *bri1* extends beyond the root response to BRs. Like *bri1*, *cu-3* is an extreme dwarf with dark-green, curled leaves that form a mass not more than 2.5 cm in either dimension (Fig. 2, C–G; Yu, 1982). Both mutants exhibit reduced fertility and show a delay in developmental programs, with the *cu-3* mutant documented to live as long as 3 years (Yu, 1982).

The *BRI1* gene has recently been cloned and shown to encode a putative Leu-rich receptor kinase that is likely to be involved in BR signal transduction (Li and Chory, 1997). Genetic screens for BR insensitivity in Arabidopsis have so far yielded only alleles of this single gene (Clouse and Feldmann, 1999), suggesting that BRI1 is the only unique component of the BR signal transduction pathway or that mutations in other genes involved in BR signal transduction are lethal. Based on the extensive phenotypic and physiological similarities of *cu-3* and *bri1*, it is possible that the *CU-3* gene is the *L. pimpinellifolium* homolog of Arabidopsis *BRI1*. However, in general, insensitive mutants may also result from lesions in genes encoding numerous steps in the signal transduction pathway and occasionally from alterations in uptake, metabolism of the exogenous compound, and transport. Cloning of the *CU-3* gene will be an essential step in resolving its role in BR insensitivity. If indeed CU-3 proves to be a BRI1 homolog, sequence comparison between the two species will be of value in delineating conserved regions in the ligand binding and kinase domains that may be essential for function.

Another mutant with similar phenotype to *cu-3* and *dpy* is *dwarf*, and particularly its extreme allele, *d^x*, showing severe dwarfism and dark-green, rugose curly leaves (Rick, 1954; Hernandez-Bravo, 1967). The *DWARF* gene was found to encode a Cyt P-450 (Bishop et al., 1996) and recent work using the *d^x* allele has shown that the DWARF enzyme is responsible for the conversion of 6-deoxocastasterone to castasterone (Bishop et al., 1999). We also found in our screen of tomato mutants that *d^x* is rescued by castasterone and brassinolide treatment, confirming the results of Bishop et al. (1999; data not shown). Both *dpy* and *dwarf* have been mapped to a similar location on the long arm of chromosome 2 (Tanksley and Mutschler, 1990), but allelism tests (Hernandez-Bravo, 1967; Yu, 1988) showed that *dwarf*, *dpy*, and *cu-3* were all different loci. *cu-3* has not been placed on the classical map but is not linked to either *dwarf* or *dpy*.

In summary, we have identified the naturally occurring tomato mutants *dpy* and *cu-3* as BR deficient and insensitive, respectively. The phenotypes of these mutants closely parallel those of Arabidopsis BR mutants, and provide genetic evidence that BRs are critical for normal development of tomato, an important crop plant. Our data further support the growing body of evidence that BRs are essential for the proper expression of several developmental

programs, including cell elongation, fertility, and leaf morphogenesis. Cloning of the *DPY* and *CU-3* genes will add to the array of molecular tools becoming available for analysis of BR biosynthesis and signal transduction.

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