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Transcription of the *Arabidopsis CPD* gene, encoding a steroidogenic cytochrome P450, is negatively controlled by brassinosteroids

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Summary

The *Arabidopsis CPD* gene encodes a cytochrome P450 steroid side-chain hydroxylase (CYP90) that plays an essential role in the biosynthesis of the plant hormone brassinolide. Expression of the *CPD* gene is confined to cotyledons and leaf primordia in etiolated seedlings and detectable in the adaxial parenchyma of expanding leaves in light-grown plants. Transcription of the *CPD* gene is not affected by the plant growth factors auxin, ethylene, gibberellin, cytokinin, jasmonic acid and salicylic acid, but is specifically down-regulated by brassinolide in both dark and light. Steady-state mRNA levels of a *CPD* promoter-driven *uidA* reporter gene correlate with the expression of resident *CPD* gene in transgenic plants. Intermediates of the early and late C-6 oxidation pathways of brassinolide, carrying C-22 and C-23 side-chain hydroxyls, efficiently inhibit the activity of the *CPD* promoter. Repression of *CPD* transcription by brassinosteroids is sensitive to the protein synthesis inhibitor cycloheximide, indicating a requirement for *de novo* synthesis of a regulatory factor.

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Introduction

Steroid hormones play an important role in the regulation of differentiation, sex determination, and maintenance of body homeostasis in animals. The biosynthesis of animal steroid hormones requires at least six different cytochrome P450 genes, the expression of which is tightly regulated by signalling mechanisms safeguarding cholesterol homeostasis (Honda *et al.*, 1993; Waterman and Bischof, 1997). High cholesterol levels in animals enhance the synthesis of oxidized cholesterol derivatives termed oxysterols. In turn, oxysterols induce an end-product repression of genes involved in steroid biosynthesis (Brown and Goldstein, 1997; Goldstein and Brown, 1990).

Recently, genetic analysis of *Arabidopsis* and garden pea mutants has provided unequivocal evidence that brassinosteroids (BRs) are essential phytohormones (Clouse, 1996; Hooley, 1996). Brassinolide, a product of campesterol oxidation, is required for the regulation of cell elongation, stress responses, male fertility, pigment biosynthesis, and numerous other developmental and physiological responses in higher plants (Mandava, 1988; Sakurai and Fujioka, 1993, 1997). Our current knowledge on the control of biosynthesis and molecular action of BRs is largely based on studies of *Arabidopsis* mutants *det2*, *cpd*, *dim* and *bri1* (Clouse, 1997; Fujioka and Sakurai, 1997a; Sasse, 1997; Yokota, 1997). *DET2* encodes a steroid 5α-reductase catalysing the synthesis of (24*R*)-24-methyl-5α-cholestan-3-one from (24*R*)-24-methylcholestan-4-en-3-one (Fujioka *et al.*, 1997). In transgenic human cells, DET2 mediates the conversion of progesterone, testosterone and androstenedione to their 5α-reduced forms, suggesting a remarkable conservation of plant and animal steroidogenic enzymes (Li *et al.*, 1996, 1997). DIM1 is thought to control an early step in BR biosynthesis affecting the expression of a β-tubulin gene (Takahashi *et al.*, 1995). *CPD* encodes a cytochrome P450 (CYP90) required for C-23 hydroxylation of cathasterone to teasterone (Kauschmann *et al.*, 1996; Szekeres *et al.*, 1996). *BRI1* codes for a putative receptor kinase carrying an N-terminal endoplasmic reticulum localization signal followed by leucine-rich repeats essential for BR perception (Li and Chory, 1997). Molecular analysis of these mutants indicates that deficiencies in BR biosynthesis and perception not only cause dramatic

phenotypic alterations, but also induce significant changes in gene expression leading for example to the activation of certain light-induced genes in the dark and de-repression of many stress-regulated genes in the light (Kauschmann *et al.*, 1996; Li *et al.*, 1996; Szekeres *et al.*, 1996). Characterization of BR-induced genes suggests that BRs may control gene expression either at the transcriptional (Xu *et al.*, 1995) or post-transcriptional level (Zurek and Clouse, 1994).

Here we demonstrate that side-chain hydroxylated BRs, including brassinolide, negatively regulate the transcription of the *CPD* steroid hydroxylase gene in *Arabidopsis*. Down-regulation of *CPD* transcription by BRs is inhibited by cycloheximide. The data suggest an intriguing analogy between end-product control of genes involved in steroid biosynthesis in plants and animals.

Results

Transcription of the CPD *gene is repressed by brassinolide*

Recently, it has been found that the *Arabidopsis CPD* gene encodes a cytochrome P450 (CYP90) showing sequence similarity to animal steroid hydroxylases. Feeding of the *cpd* mutant with biosynthetic precursors of brassinolide indicated that CYP90 may catalyse the conversion of cathasterone to teasterone by C-23 hydroxylation (Szekeres *et al.*, 1996). The *CPD* gene appeared thus to control an essential step in brassinolide biosynthesis. To determine whether BR biosynthesis is regulated at the level of *CPD* gene expression, a 965 bp DNA segment, carrying the *CPD* promoter and 5′-untranslated leader sequences, was cloned upstream of an *uidA* reporter gene in the promoter test vector pPCV812 (Koncz *et al.*, 1994). Following introduction of the reporter gene construct into *Arabidopsis* plants, RNA hybridizations were performed to compare the levels of steady-state mRNAs synthesized from the resident *CPD* gene and the *CPD* promoter-driven *uidA* reporter gene (Figure 1). The levels of *CPD* and *uidA* mRNAs were comparable in both dark- and light-grown transgenic seedlings, indicating a potential for use of the *CPD* promoter–*uidA* reporter construct in gene expression studies.

To search for factors regulating *CPD* gene expression, preliminary assays were carried out by monitoring the GUS reporter enzyme levels in seedlings exposed to different growth hormones in liquid MS medium (Figure 2c). Auxin (indole-3-acetic acid, IAA and α-naphthaleneacetic acid, NAA), gibberellin (GA_3), and cytokinin (6-benzylaminopurine, BAP) did not affect significantly the levels of GUS enzyme produced by the *CPD* promoter–*uidA* reporter construct. Methyl-jasmonate, salicylic acid, 1-aminocyclopropane-1-carboxylic acid (an ethylene precursor) and ergosterol (a fungal steroid) also proved to be inactive in

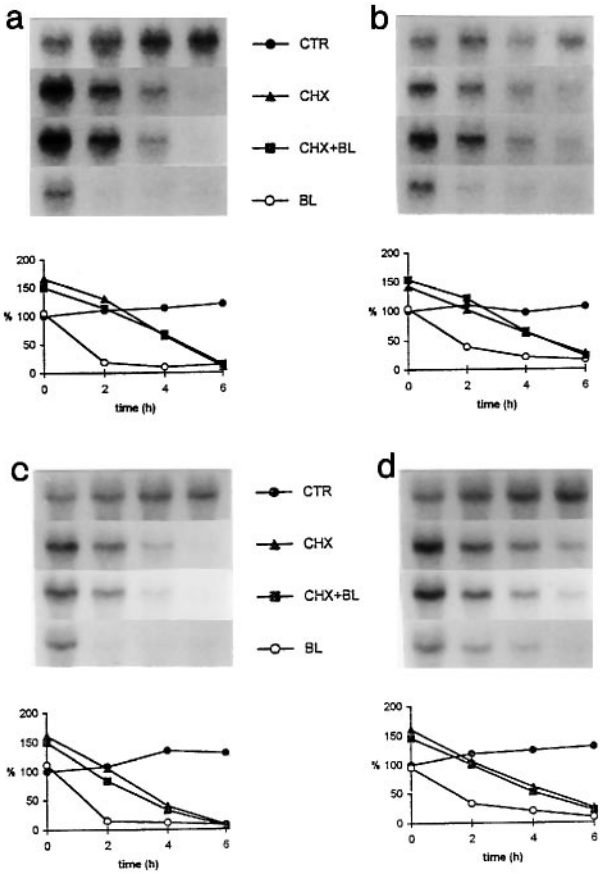

Figure 1. Transcription of the *CPD* gene and *CPD* promoter-driven *uidA* reporter gene is down-regulated by brassinolide in a cycloheximide-dependent fashion in transgenic plants.

Seedlings were grown for 8 days in the light (a,c) or in the dark (b,d), then transferred into liquid MS medium. Brassinolide was added by dilution of 1 mM stock in ethanol at a final concentration of 1 μM (BL), whereas control samples were treated with 0.1% ethanol (CTR) or with 100 μM cycloheximide (CHX). To monitor the effect of inhibition of protein synthesis, seedlings were treated with 100 μM cycloheximide before the addition of 1 μM brassinolide (CHX + BL). Samples for RNA preparation and Northern blotting were collected after 0, 2. 4 and 6 h of treatment. Equal loading of RNA blots was monitored by hybridization with a control probe as described in Experimental procedures, then the blots were hybridized with *CPD* (a,b) and *uidA* (c,d) probes. Quantitative hybridization data are plotted as percentage of the zero-time control values.

these assays (data not shown). However, brassinolide treatment of the seedlings resulted in a drastic reduction of GUS enzyme level, suggesting that the activity of the *CPD* promoter may be controlled by the end-product of BR biosynthesis. Titration assays showed that the inhibitory effect of brassinolide is concentration-dependent. Concentrations as low as 1 nM brassinolide resulted in 50% inhibition, and a maximum level of about 90% reduction of GUS activity was detected in seedlings exposed to brassinolide concentrations higher than 0.1 μM (Figure 2a).

To confirm that BR-mediated down-regulation of the *CPD* promoter–*uidA* reporter indeed resulted from a repression of *CPD* transcription, RNA samples were prepared from etiolated and light-grown seedlings incubated with 1 μM

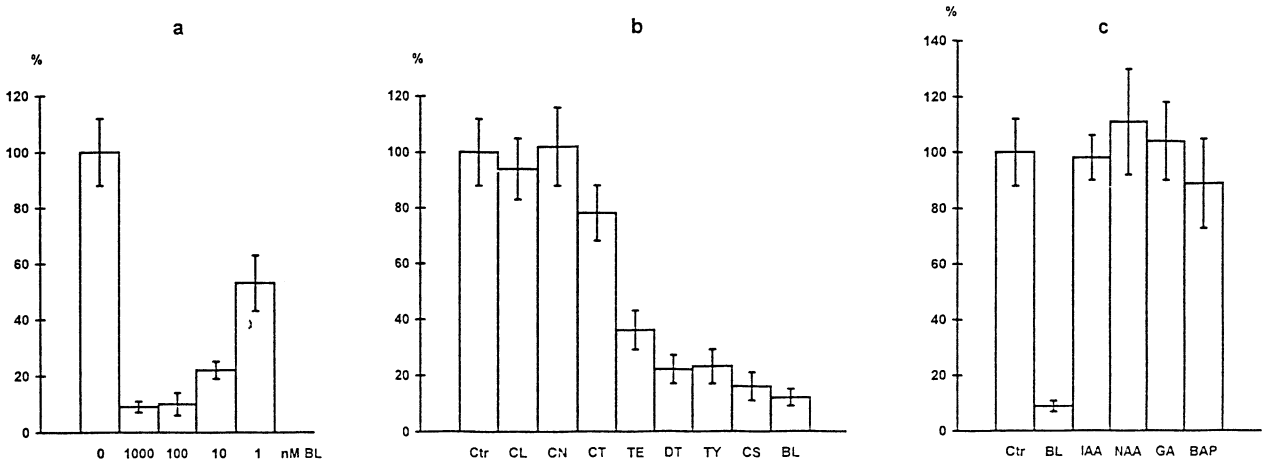

Figure 2. Effects of phytohormones on the activity of the *CPD* promoter-driven *uidA* reporter gene in *Arabidopsis*.
Pools of 20 seedlings were grown for 8 days under light/dark cycles in MS medium with brassinolide (a), brassinolide precursors (b), and different phytohormones (c) for spectrofluorimetric determination of GUS enzyme activities. Seedling were grown (a) in the presence of 0, 1, 10, 100 and 1000 nM brassinolide, (b) on MS medium (Ctr), MS medium containing 1 μM campesterol (CL), campestanol (CN), cathasterone (CT), teasterone (TE), 3-dehydroteasterone (DT), typhasterol (TY), castasterone (CS) or brassinolide (BL) and (c) on MS medium (Ctr), MS medium containing 1 μM brassinolide (BL), indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), gibberellic acid (GA), or 6-benzylaminopurine (BAP). The data are means of three independent experiments with standard deviation. All data are expressed as a percentage of the untreated control values.

brassinolide for 0, 2, 4 and 6 h with or without previous treatment with 100 μM cycloheximide for 30 min (Koshiba *et al.*, 1995). Control samples were incubated with or without cycloheximide in MS medium. To simultaneously detect temporal changes induced by brassinolide and cycloheximide in the expression of resident *CPD* gene and the *CPD* promoter–*uidA* reporter construct, the RNA samples were subjected to Northern hybridization using the *CPD* cDNA and *uidA* coding region as probes. Exposure of light-grown seedlings to brassinolide reduced the *CPD* RNA level to 10% of the untreated control within 2 h and no further decrease was seen following 4 and 6 h of treatment (Figure 1). Brassinolide-induced decrease of the steady-state *CPD* RNA level was slightly slower in dark-grown seedlings. The temporal expression pattern of the *CPD* promoter-driven *uidA* reporter gene, carrying 3′-untranslated sequences from the nopaline synthase gene (Koncz *et al.*, 1994), showed a remarkable similarity to that of the *CPD* gene. In etiolated seedlings, the *uidA* mRNA levels gradually decreased during the 6 h of brassinolide treatment, whereas the *uidA* mRNA was barely detectable in light-grown seedlings after 2 h of exposure to brassinolide.

Repression of CPD *transcription by brassinolide requires* de novo *protein synthesis*

Inhibition of protein synthesis by cycloheximide for 30 min resulted in a transient increase in the *CPD* and *uidA* RNA levels, which was followed by a gradual decrease during the 6 h incubation period. Depletion of both transcript pools was somewhat faster in light-grown seedlings than in etiolated ones: after 6 h their respective values were about 10% and 20% of their initial levels (Figure 1). Whereas treatment of the plants with brassinolide for 2 h caused a substantial inhibition of *CPD* and *uidA* gene expression both in light and dark, in plants incubated with cycloheximide for 30 min and then with brassinolide this effect was completely abolished and steady-state transcript levels were identical with those detected in the cycloheximide-treated control. Therefore, the data suggested that inhibition of either protein synthesis or transcript degradation (Gil and Green, 1996) by cycloheximide could prevent a down-regulation of *CPD* transcript levels by brassinolide. The level of steady-state mRNAs derived from both *CPD* and *CPD* promoter-driven *uidA* genes showed a decrease, rather than an increase, in plants incubated for longer time periods with cycloheximide. The results thus indicated that brassinolide affected either the regulation of *CPD* promoter activity or increased non-specific turnover of unrelated *CPD* and *uidA* mRNAs. The inhibition of brassinolide-mediated down-regulation of *CPD* transcription by cycloheximide suggested a requirement for *de novo* synthesis of a negative regulatory factor.

Tissue-specific regulation of CPD *gene expression*

Pools of transgenic F_2 seedlings carrying the *CPD* promoter–*uidA* reporter construct displayed GUS enzyme activities between 1.2×10^2 and 7.6×10^3 nmol of 4-methylumbelliferyl glucuronide min^{-1} mg^{-1} protein. These pools were used for monitoring temporal and spatial activity of the *CPD* promoter in etiolated and light-grown seedlings (Jefferson, 1987). During early development,

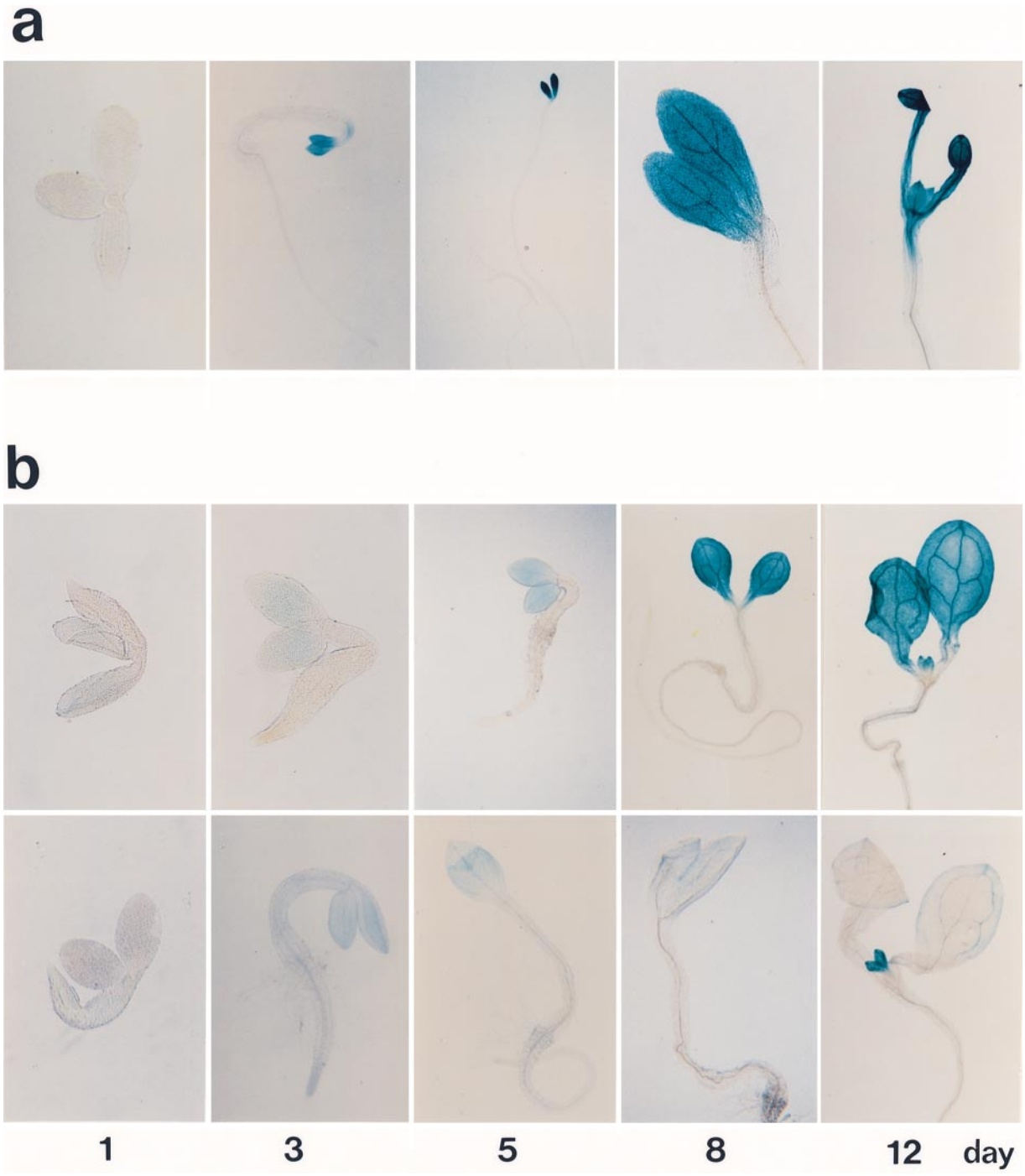

Figure 3. Developmental activation and brassinolide-mediated inhibition of the *CPD* promoter during early seedling development.
GUS histochemical staining of transgenic seedlings carrying the *CPD* promoter–*uidA* reporter construct germinated and grown for 1, 3, 5, 8 and 12 days in the dark (a) and in the light (b). The lower panel in (b) shows seedlings grown in the presence of 1 μM brassinolide.

similar GUS staining patterns were observed in both etiolated and light-grown seedlings (Figure 3). GUS activity appeared only in the cotyledons three days after imbibition and increased throughout the first week of seedling development. Occasionally, some weak GUS staining was also observed in the uppermost section of hypocotyls in light-grown plants, as well as in the apical hook of dark-grown seedlings, but never occurred in lower sections of hypocotyl or in the roots. Ten days after germination, GUS staining appeared in the leaf primordia and showed a concomitant decrease in the cotyledons. Seedlings grown in the presence of 1 μM brassinolide showed a similar

distribution but much lower level of GUS activity, and intense staining was seen only in the expanding leaf primordia (Figure 3b). At later stages of plant development in the light, the GUS staining was confined to leaf buds and young rosette leaves, but was absent in petioles. In ageing leaves, the GUS activity gradually decreased and became restricted to the leaf margins. Leaf-blade cross-sections showed a high GUS activity in mesophyll cells, especially those of the palisade parenchyma, and the stomatal guard cells of adaxial epidermis (Figure 4). In the inflorescence, GUS activity was found in cauline leaves and sepals. No GUS staining was observed in other flower organs, siliques, peduncles and mature roots (Figures 3 and 4).

Feed-back regulation of CPD *promoter by products of phytosterol oxidation pathways*

Developmental activation of the *CPD* promoter appears to be independent of light signalling because similar levels of *CPD* and *uidA* transcripts (Figure 1), as well as identical GUS staining of cotyledons (Figure 3), were detected in seedlings grown for 8 days in the dark and light. Thus, GUS enzyme activities measured in young seedlings seemed to correlate with the activity of the *CPD* promoter. To test whether the GUS reporter is indeed suitable for monitoring BR-induced down-regulation of the *CPD* promoter, seedlings were germinated in the presence or absence of 1 μM brassinolide for 8 days in both dark and light. Histological staining (Figure 3) and fluorimetric enzyme assays (Figure 2c) showed that brassinolide treatment caused a 90% reduction of GUS enzyme activity in seedlings grown in both dark (data not shown) and light.

To search for further compounds regulating the *CPD* promoter, three pools of 20 seedlings were grown for 8 days with BRs added at 1 μM concentration, then subjected to GUS assays using untreated seedlings as control. Except for campesterol and campestanol, intermediates of the early C-6 oxidation pathway of brassinolide biosynthesis, including cathasterone, teasterone, 3-dehydroteasterone, typhasterol and castasterone resulted in a significant reduction of *CPD* promoter-dependent GUS activity (Figure 2b). The extent of *CPD* inhibition observed with these BRs correlated well with their biological activity detected in other bioassays (Fujioka *et al.*, 1995, 1997). A reduction of GUS activity was analogously detected with BR intermediates of the late C-6 oxidation pathway (Sakurai and Fujioka, 1997). In comparison to BRs involved in the early C-6 oxidation pathway, their 6-deoxo derivatives and *epi*-stereoisomers showed lower activities. Changes in the stereoisomery of side-chain substitutions and the lack of C-6 oxidation also reduced, but did not abolish, the *CPD* inhibitory activity of BRs derived from campesterol (Figures 5 and 6). Remarkably, some rare BRs, such as 28-norcastasterone, 28-homobrassinolide, 28-homodolicholide, 28-homodolichosterone, and 22,23-*diepi*-28-homobrassinolide displayed in contrast a very significant inhibitory effect. Although 24-*epi*-teasterone proved to be inactive, neither the presence nor the type of C-24 substitution appeared to be absolutely required for BR-induced inhibition of the *CPD* promoter. The absence or presence of C-2 hydroxylation proved to be even less important, as typhasterol and castasterone which differ at this position showed similar activities (Figures 2b and 5). However, the fact that ergosterol and cholesterol showed no activity in the assay indicated that 5α-reduction as well as 22- and/or 23-hydroxylation of the side-chain may be essential for efficient feedback inhibition of *CPD* gene expression.

Discussion

The BR-deficient phenotype of the *cpd* mutant (Szekeres *et al.*, 1996) indicates that in *Arabidopsis* there is no alternative to CYP90-mediated C-23 hydroxylation of the BR side-chain. Brassinolide-mediated inhibition of *CPD* gene activity attests to the existence of a negative feedback regulation that controls BR synthesis at this enzymatic step. Since gibberellin biosynthesis is also modulated by a similar mechanism involving expressional regulation of GA 20-oxidases (Phillips *et al.*, 1995), feedback control seems to represent a mode of self-regulation of phytohormone levels. Whereas our experiments with biosynthetic congeners of brassinolide identified teasterone, the putative product of CYP90, as the first intermediate eliciting substantial inhibitory effect, we also observed a significant (20%) decrease of *CPD* promoter–*uidA* expression in transgenic seedlings treated with cathasterone. However, it is currently unknown whether intermediates of the early and late C-6 oxidation pathways of brassinolide are themselves active as hormones or need to be converted to brassinolide, representing the only active steroid hormone. The activity of brassinolide precursors repressing the *CPD* promoter may therefore solely reflect their position and rate of conversion in the brassinolide biosynthesis pathways (Sakurai and Fujioka, 1997). Nonetheless, the *CPD* inhibitory activity of BRs derived from phytosterols other than campesterol suggests that either other functional steroid hormones synthesized from, e.g. sitosterol, isofucosterol or 24-*epi*-campesterol (Yokota, 1997) exist in plants or that each BR compound is hormonally active on its own, or both. Whereas the first possibility is supported by detection of sitosterol and isofucosterol-derived compounds in several plant species (Adam *et al.*, 1996; Fujioka and Sakurai, 1997b; Sakurai and Fujioka, 1993, 1997) and the observation that 24-*epi*-brassinolide is the only detectable BR form in *Gypsophila perfoliata* seeds (Schmidt *et al.*, 1997), testing of the second possibility would require the characterization of ligand-

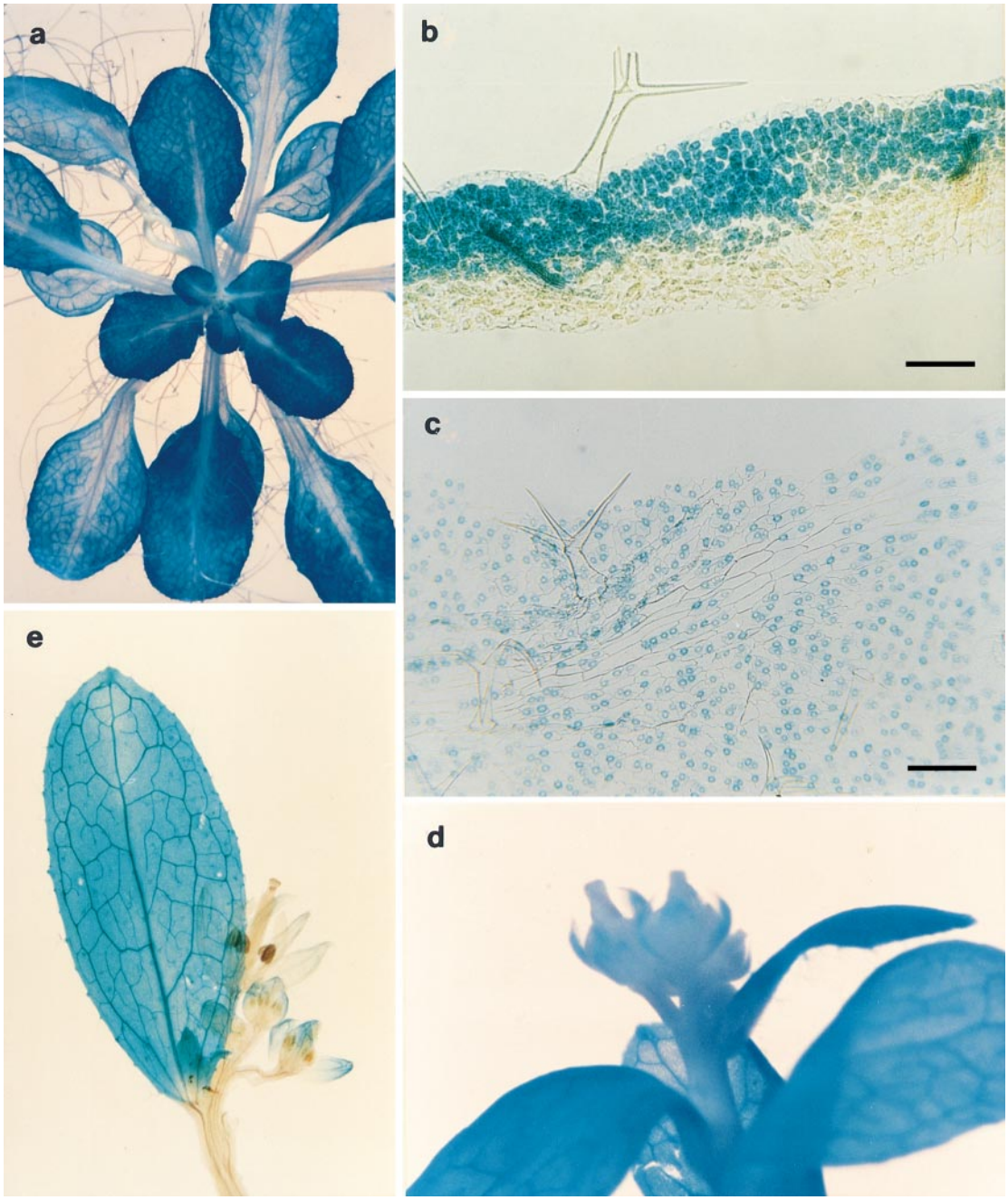

Figure 4. Tissue-specific distribution of GUS activity in *Arabidopsis* plants carrying the *CPD* promoter–*uidA* reporter gene construct after 4 weeks of development in the light.
(a) Rosette leaves and roots, (b) leaf-blade cross-section, (c) epidermal peel from the upper leaf surface, (d,e) flowers and cauline leaves.
Scale bars represent 100 μm.

binding properties of plant steroid receptors. The recent finding that *BRI1* codes for a potential transmembrane leucine-rich repeat (LRR)-receptor kinase required for steroid perception may therefore facilitate the functional analysis of different BR compounds (Li *et al.*, 1997).

Apart from exploring possible action mechanisms of plant steroid hormones, the data showing BR-dependent feedback inhibition of *CPD* transcription are intriguing because they suggest an analogy between end-product regulation of genes involved in animal and plant steroid biosynthesis. In animals, oxidized derivatives of cholesterol carrying one or more hydroxyl groups at positions 7, 20, 22, 25 or 27 regulate both steroidogenic factors (i.e. nuclear steroid receptors) and membrane-bound sterol regulatory element-binding proteins (SREBPs) controlling the activity of genes involved in steroid biosynthesis. The structure of

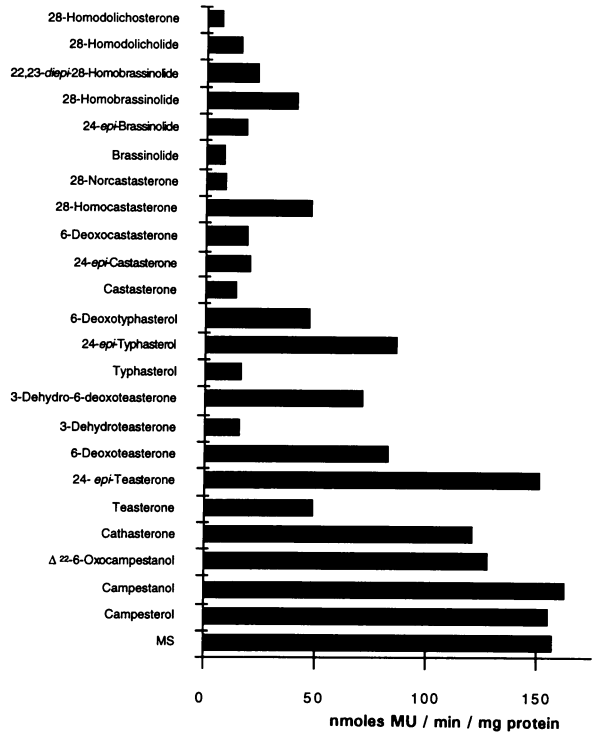

Figure 5. Screening for brassinosteroids inhibiting the *CPD* promoter activity.
Specific GUS activities were determined after growing *Arabidopsis* seedlings carrying the *CPD* promoter-driven *uidA* reporter construct in the dark for 8 days in MS medium (MS) or in MS medium containing 1 μM of the brassinosteroids listed at the left and in Figure 6. The data represent an experiment performed with three pools of 20 seedlings.

these animal oxysterols differs from those of BRs by the lack of 5α-reduction and C-2 hydroxylation. It may be worth noting a similarity between oxysterols and BRs, because functional conservation of 5α-steroid reductases has been demonstrated between plants and animals (Li *et al.*, 1997) and because our data indicate that C-2 hydroxylation of BRs is probably not essential for *CPD* repression. A further observation pointing to an analogy between sterol feedback regulation in plants and animals is that both oxysterol-induced transcriptional repression in animals and BR-mediated inhibition of *CPD* transcription in *Arabidopsis* are sensitive to the protein synthesis inhibitor cycloheximide. As known for oxysterol signalling (Brown and Goldstein, 1997; Waterman and Bischof, 1997), feedback regulation of *CPD* by BRs seems to require the synthesis or processing (or both) of a negative regulatory factor, which may directly or indirectly control *CPD* transcription.

To confirm this model, a well-defined 965 bp promoter region is now available for mapping of *cis*-regulatory sequences controlling *CPD* transcription in response to BRs. We have shown that BRs repress developmental activation of the *CPD* promoter in cotyledons during early seedling development and that BR-induced reduction of *CPD* transcript levels occurs faster in the light than in the dark. The fact that the *CPD* promoter–*uidA* reporter construct was found to be active in defined cell types of leaves also suggests that the synthesis and/or stability of *CPD* mRNA may be controlled in a complex fashion by light and/or developmental signals. Our data regarding developmental activation of the *CPD* gene raise some immediate questions. Namely, if *CPD* controlled an essential step of brassinolide biosynthesis, and brassinolide was required for promotion of hypocotyl elongation, why is the *CPD* gene expression confined to cotyledons and not detectable in actively elongating cells throughout the hypocotyls and roots? Also, if the highest brassinolide levels were detected in pollen and seeds (Mandava, 1988), why is the *CPD* gene not expressed in these tissues? In fact, if BRs were the sole regulators of the *CPD* gene, the absence of *CPD* gene expression would indicate that the concentration of BRs in these tissues is high enough to repress *CPD* transcription. However, this model would predict that steroid hormones are efficiently transported from source to target tissues in the plant and/or that BRs are inactivated in the source tissues where the *CPD* gene (and BR biosynthesis) is active. Translocation of 28-homobrassinolide from the roots to leaves (Schlagnhaufer and Arteca, 1991), as well as metabolic inactivation of 24-*epi*-brassinolide by C-25 hydroxylation and subsequent glucosylation (Hai *et al.*, 1995), were in fact detected in different species. Further isolation of mutants showing changes in tissue-specific regulation of the *CPD* promoter-driven *uidA* reporter construct may help to clarify whether these mechanisms indeed play a role in the maintenance of steroid homeostasis in plants.

Experimental procedures

Plant material and growth conditions

Seeds from wild-type *Arabidopsis thaliana* (ecotype Columbia, Col-1) and derived transgenic plants were surface-sterilized and germinated on MS medium (Murashige and Skoog, 1962) supplemented with 0.5% sucrose and 0.2% Phytagel (Sigma Chemical Co.) at 22°C, either in the dark or under 14 h light/10 h dark cycles, as described by Koncz *et al.* (1994). Seedlings were grown for 8 days in the presence of 1 or 10 μM indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), gibberellic acid (GA_3), 6-benzylaminopurine (BAP), methyl-jasmonate (JA), salicylic acid (SA), 1-aminocyclopropane-1-carboxylic acid (ACC) or ergosterol to screen for factors affecting *CPD* promoter activity. The biosynthetic congeners of brassinolide used in the experiments were chemically synthesized (Fujioka *et al.*, 1997).

Construction of transgenic plants carrying a CPD *promoter-driven* uidA *reporter gene*

The *CPD* promoter region, extending from a *Bam*HI site at position –919 to a PCR-generated *Sma*I site at position +45 (GenBank Accession No. X87368), was PCR-amplified, cloned in pBluescript

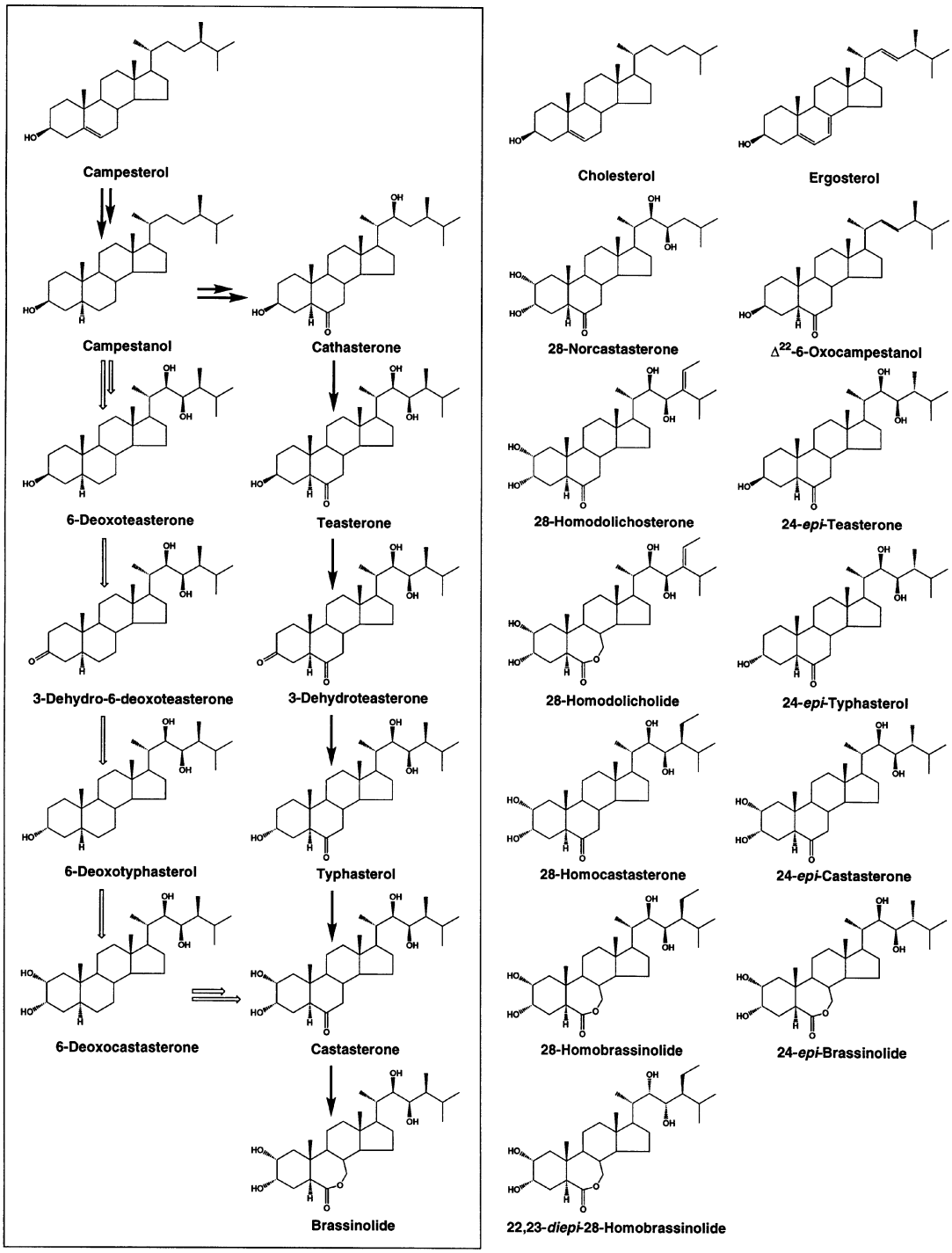

Figure 6. Chemical structures of brassinosteroids used in the *CPD* promoter-based bioassays.
Intermediates of the brassinolide pathway and their biosynthetic relationships are shown in the box. Conversion steps of the early and late C-6 oxidation pathways are indicated by solid and open arrows, respectively. Double arrows represent more than one enzymatic reaction.

(Stratagene), and sequenced using a PCR kit (ABI Prism™ Dye Terminator Cycle Sequencing) and an automatic DNA sequencer (ABI 377). The *Bam*HI–*Sma*I *CPD* promoter fragment was cloned into *Bgl*II–*Sma*I sites of a polylinker located upstream of the promoterless *uidA* reporter gene in the promoter test vector pPCV812 (Koncz *et al.*, 1994). The resulting construct was transferred to *Agrobacterium* GV3101 (pMP90RK) and transformed into *Arabidopsis* as described by Koncz *et al.* (1994). From transformed root cultures, transgenic plants were regenerated by selecting for the hygromycin resistance marker of pPCV812 T-DNA. After testing the GUS activity in 12 independent transformant lines, seeds from the selfed F_2 families carrying the T-DNA of the pPCV812 construct

were screened for by germinating seeds on MS medium containing 15 mg l^{-1} hygromycin, then bulked for RNA isolation and GUS assays.

Hybridization analysis of steady-state RNA levels

Seedlings were germinated and grown on Phytagel-supported medium for 8 days in the dark or light, then transferred to liquid medium and treated for 2, 4 and 6 h with 100 μM cycloheximide or 1 μM brassinolide (CIDtech Research Inc., Missisauga, Ontario, Canada) under the same light conditions. Other samples were pre-incubated with 100 μM cycloheximide to stop protein synthesis as described by Koshiba *et al.* (1995), then treated with 1 μM brassinolide for 2, 4 and 6 h. Control samples were incubated in liquid medium for similar time periods. Aliquots of 25 μg total RNA isolated as described by Nagy *et al.* (1988) were subjected to Northern blotting (Sambrook *et al.*, 1989) with ^{32}P-labelled probes generated by random priming using the full-length *CPD* cDNA (GenBank Accession No. X87367) or the *uidA* coding region (GenBank Accession No. X02395). Equal loading of filters was controlled as described earlier (Szekeres *et al.*, 1996). Following hybridization, the RNA filters were washed twice with 2× SSC, 0.05% SDS, and twice with 0.1× SSC, 0.1% SDS at 65°C as described previously (Sambrook *et al.*, 1989), subjected to autoradiography and evaluated quantitatively using a PhospholmagerTM 445 SI image analyser (Molecular Dynamics Inc., Sunnyvale, CA).

Histochemical and fluorimetric GUS enzyme assays

Histochemical localization of GUS activity was carried out according to Jefferson (1987) with some modifications. Before incubation with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc), the samples were fixed for 10 min in a 2% formaldehyde solution as described by Puente *et al.* (1996). Uptake of the staining solution was facilitated by a 10 sec pulse of reduced pressure and verified by control staining of transgenic seedlings ubiquitously expressing the GUS reporter. Following the staining procedure, the samples were washed with 70% ethanol to remove plant pigments. For microscopic examination, leaves were hand-sectioned following the staining process. Spectrofluorimetric assay of GUS activity was performed as described previously (Jefferson, 1987) using 4-methyl-umbelliferyl-β-D-glucuronide (MUG) as substrate. GUS enzyme activity was measured at four time points to verify linearity of the reactions. To monitor the effect of phytosterol derivatives repressing the *CPD* promoter–*uidA* reporter construct, three pools of 20 seedlings were grown in the dark or light at 22°C for 8 days in MS medium containing 1 μM of the sterol compounds listed in Figure 6, and subjected to fluorimetric GUS assays. For GUS histochemical analysis, seedlings were grown for 1, 3, 5, 8 or 12 days in the presence or absence of phytosterols under similar conditions, then fixed and treated with staining solution (1 mg ml^{-1} X-gluc in 50 mM pH 7.0 sodium phosphate buffer containing 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide) for 12 h. Untreated control samples used for RNA analysis or GUS assay received the same amount of ethanol as was carried over by addition of BR stock solutions.

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