CYP72B1 Inactivates Brassinosteroid Hormones: An Intersection between Photomorphogenesis and Plant Steroid Signal Transduction[1]

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Active brassinosteroids, such as brassinolide (BL) and castasterone, are growth promoting plant hormones. An Arabidopsis cytochrome P450 monooxygenase encoded by *CYP72B1* has been implicated in brassinosteroid catabolism as well as photomorphogenesis. We expressed CYP72B1 in yeast, coupled with brassinosteroid feeding, and established the biochemical function to be the hydroxylation of BL and castasterone, to give 26-hydroxybrassinolide and 26-hydroxycastasterone, respectively. Brassinosteroid feeding experiments with wild-type Arabidopsis, a *CYP72B1* null mutant, and a *CYP72B1* overexpression line demonstrated that carbon 26 hydroxylation of active brassinosteroids is an endogenous function of CYP72B1. Seedling growth assays demonstrated that 26-hydroxybrassinolide is an inactive brassinosteroid. Genetic and physiological analysis of the hypocotyl response to exogenous BL and varying intensities of white and monochromatic light suggested that *CYP72B1* modulates photomorphogenesis primarily through far-red light and to a lesser extent through blue- and red-light pathways. *CYP72B1* transcript accumulation in dark-grown seedlings was organ specific and down-regulated after 1 h of illumination in dim white, red, and blue light, but not far-red light. CYP72B1 translational fusions with the β-glucuronidase reporter gene demonstrated that protein levels increased in the hypocotyl elongation zone when shifted from the dark to far-red light, but not blue or red light. We propose a model in which Arabidopsis seedling development switches from dark-grown development (skotomorphogenesis) to light-grown development (photomorphogenesis) in part by rapid modulation of brassinosteroid sensitivity and levels. CYP72B1 provides an intersection between the light and brassinosteroid pathways mainly by far-red-light-dependent modulation of brassinosteroid levels.

Brassinolide (BL) is the most active brassinosteroid, a class of polyhydroxylated plant-specific steroids. The isolation of BL in 1979 identified the structure to be a cholestane derivative (Grove et al., 1979). Animal steroids are likewise cholestane derivates (Mussig and Altmann, 2001). Analysis of BL biosynthetic mutants (*det2* and *cpd*) in Arabidopsis revealed similarities between animal and plant steroids. Rescue of the *det2* and *cpd* pleiotropic phenotypes by exogenous BL established the commonality of steroids as fundamental hormones in both animal and plant development (Li et al., 1996; Szekeres et al., 1996). Cloning of the *CPD* gene from Arabidopsis demonstrated that animals and plants each use cytochrome P450 monooxygenases (CYP450s) for steroid biosynthesis (Szekeres et al., 1996). Analysis of the human steroid 5α-reductase (hS5R) and its Arabidopsis ortholog, DET2, demonstrated a common mechanism of steroid hormone activation between animals and plants (Li et al., 1996). Both human isoenzymes of hS5R reduce testosterone to dihydrotestosterone to amplify a weak hormone signal, whereas DET2 reduces BL precursors (Fujioka et al., 2002). Ecdysone, an insect steroid hormone, is structurally similar to BL and inactivated by carbon 26 hydroxylation via the action of an unidentified CYP450 (Williams et al., 2000). An analogous inactivation of BL was suggested when overexpression of *BAS1/CYP72B1*, a putative Arabidopsis CYP450, resulted in Arabidopsis (*bas1*-D) and tobacco plants with BL-deficient-like phenotypes and heightened accumulation of 26-hydroxybrassinolide (26-OHBL; Neff et al., 1999).

Although many animal steroid hormones have been firmly established as components of endocrine systems with well-characterized roles in animal development, the role of brassinosteroids in plant development is less clear. BL can affect cell elongation, division, and differentiation, thus defects in brassinosteroid synthesis or sensing are likely to affect many plant development pathways (Friedrichsen and Chory, 2001). Recent molecular studies in Arabidop-

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sis, pea (*Pisum sativum*), and tomato (*Lycopersicon esculentum*) indicate that promotion of cell expansion and regulation of photomorphogenesis are among the most important roles of brassinosteroids (Szekeres and Koncz, 1998; Clouse and Feldmann, 1999; Mussig and Altmann, 1999; Bishop and Yokota, 2001).

Brassinosteroid-deficient or -insensitive mutants as well as Arabidopsis seedlings treated with a brassinosteroid biosynthesis inhibitor (Brz) have light-grown phenotypes when grown in the dark (Fankhauser and Chory, 1997; Asami and Yoshida, 1999; Nagata et al., 2000). These observations lead to the hypothesis that light may alter either the concentration of brassinosteroids or the responsivity of cells to these steroids (Fankhauser and Chory, 1997). Recent microarray analysis of brassinosteroid-regulated genes in Arabidopsis lead to the hypothesis that brassinosteroids may act as regulators of the light-signaling pathway rather than functioning as downstream mediators of light signal transduction (Goda et al., 2002). Research measuring levels of castasterone (CS), another active brassinosteroid, does not support a role for differential brassinosteroid levels in pea de-etiolation (Symons and Reid, 2003). Brassinosteroid levels are slightly increased in pea epicotyls in response to light, whereas three brassinosteroid biosynthetic mutants in pea are not de-etiolated in the dark (Symons et al., 2002). In contrast, the Pra2 small GTPase from pea is dark-induced and presumably modulates brassinosteroid content through a GTP-dependent interaction with DDWF1, a brassinosteroid biosynthetic protein (Kang et al., 2001). It remains unclear whether modulation of endogenous brassinosteroid levels or sensitivity provides a mechanism by which light cues are integrated into this hormone pathway.

A previous genetic characterization using overexpression and antisense lines suggested that *BAS1*/*CYP72B1* acts as a point of cross-talk between brassinosteroids and photomorphogenesis. Though present in wild-type adult plants, BL was undetectable in adult *bas1*-D mutants. Levels of CS were reduced in *bas1*-D plants compared with the wild type. When labeled BL was fed to *bas1*-D seedlings, a heightened accumulation of 26-OHBL was detected relative to the wild type. Together, these observations lead to the hypothesis that, in Arabidopsis, active brassinosteroids can be inactivated via CYP72B1-mediated hydroxylation at carbon 26 (Neff et al., 1999; Fig. 1). The present study confirms the biochemical function of CYP72B1 and further dissects the interaction between brassinosteroid inactivation and photomorphogenesis.

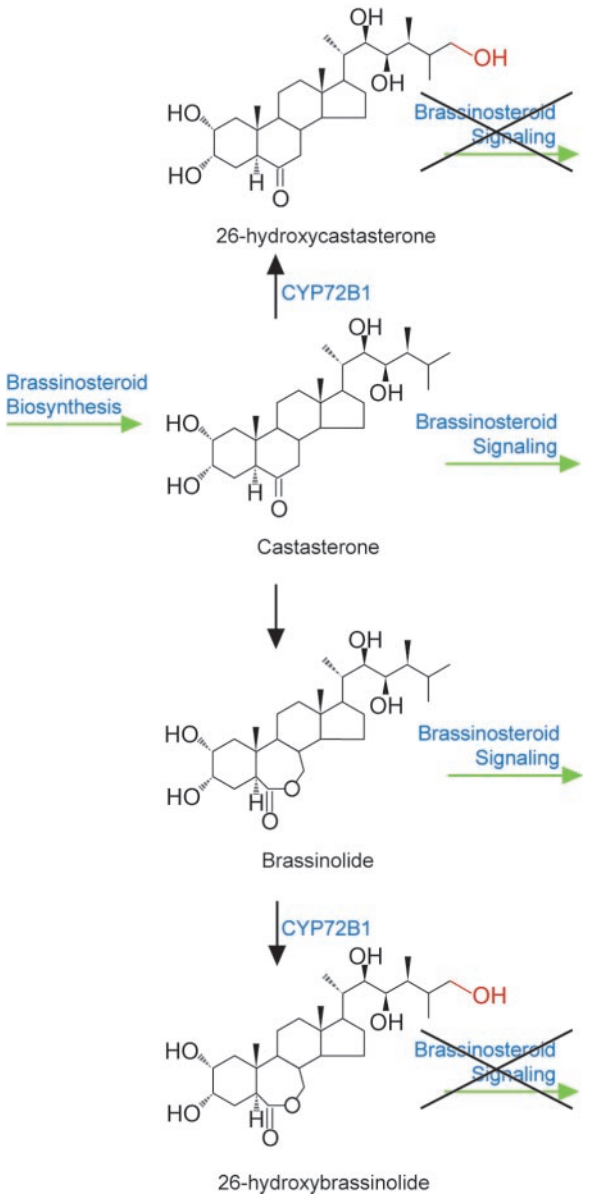


Figure 1. Catabolic pathway of active brassinosteroids. CYP72B1 has been hypothesized to inactivate growth-promoting brassinosteroids such as BL and CS via hydroxylation at carbon 26 (Neff et al., 1999).

RESULTS

CYP72B1 Is a Brassinosteroid Carbon 26 Hydroxylase

We took two complementary approaches to test the hypothesis that CYP72B1 converts BL to 26-OHBL and to examine the possibility that CS is also a substrate for this enzyme. First, we expressed *CYP72B1* cDNA in the WAT11 yeast strain using the pYeDP60 yeast-expression vector (Pompon et al., 1996). Under inductive conditions, carbon monoxide differential spectra confirmed a low level of active CYP72B1 protein (data not shown). Gas chromatography-mass spectrometry (GC-MS) was used to compare a 26-OHBL standard to the metabolite that resulted from feeding BL to yeast expressing *CYP72B1* (Table I). The 26-OHBL standard and the resulting metabolite had identical retention times and mass spectra, demonstrating that CYP72B1 protein is a steroid hydrox-

Table I. *GC-MS analysis*

Identification of 26-hydroxylated metabolites converted from BL, [$^2H_6$]BL, CS, and [$^2H_6$]CS in transformed yeast cultures and Arabidopsis seedlings. Yeast were transformed with pED8, which contains the CYP72B1 open reading frame under the control of a Gal-inducible promoter. Arabidopsis seedlings tested were the wild type (Ws-2), a CYP72B1 null (*cyp72b1-1*), and a CYP72B1 overexpressor (*cyp72b1–ox8*). All experimental replicates = 1*n*.

Compound	Yeast		Arabidopsis Seedlings		
	Retention Time	Characteristic Ions *m/z* (with Relative Intensities)	*cyp72b1-1*	Ws-2	*cyp72b1-ox8*
	min		*μg 26-OH brassinosteroid detected*		
26-OHBL standard	13.72	736 [M^+] (5), 721 (7), 619 (17), 577 (14), 564 (13), 299 (25), 156 (100)			
BL metabolite	13.72	736 [M^+] (4), 721 (6), 619 (17), 577 (15), 564 (14), 299 (27), 156 (100)	0.15	1.2	2.2
[$^2H_6$] BL metabolite	13.70	742 [M^+] (4), 727 (6), 625 (16), 583 (13), 570 (12), 299 (25), 156 (100)	0.08	1.4	2.4
26-OHCS standard	12.25	720 [M^+] (3), 705 (24), 603 (100), 514 (27)			
CS metabolite	12.25	720 [M^+] (3), 705 (23), 603 (100), 514 (29)	0.05	0.4	1.7
[$^2H_6$] CS metabolite	12.23	726 [M^+] (2), 711 (23), 609 (100), 520 (27)	0.01	0.4	1.5

ylase that can convert BL to 26-OHBL when expressed in yeast. CS, the immediate precursor to BL and itself an active brassinosteroid, was also a substrate for CYP72B1 conversion to its 26-hydroxy derivative (26-OHCS) when expressed in yeast (Table I), suggesting that CYP72B1 can have broad substrate specificity for active brassinosteroids. Empty vector transformed into WAT11 yeast showed no brassinosteroid metabolites.

To test the endogenous function of CYP72B1 in Arabidopsis, seedlings of a null mutant in this gene (*cyp72b1-1*), the wild type (Wassilewskija 2 [Ws-2]), and a transgenic line overexpressing *CYP72B1* (*cyp72b1-ox8*) were fed deuterium-labeled BL and assayed for conversion to deuterium-labeled 26-OHBL by GC-MS (Table I). A reduced level of deuterium-labeled 26-OHBL was detected in the null mutant, and an increased level was detected in the *cyp72b1-ox8* line compared with Ws-2. Feeding experiments with unlabeled BL gave qualitatively similar results. CS feeding resulted in conversion to 26-OHCS in a manner similar to BL feeding (Table I). Statistical analysis (Student's *t* test, paired, one-tailed) indicated that the wild type (Ws-2) converts each substrate to its 26-hydroxylated product more so than the *cyp72b1-1* null mutant ($P = 0.02$) and less than the *CYP72B1* overexpression line, *cyp72b1-ox8* ($P = 0.0003$). The small amount of 26-OHBL detected in the *cyp72b1-1* null mutant could be due to the action of a nonspecific or partially redundant CYP450. These genetic results confirmed the biochemical function of CYP72B1 established by the yeast functional assay and demonstrated that carbon-26 hydroxylation of the active brassinosteroids, BL and CS, is an endogenous biochemical function of CYP72B1 in Arabidopsis.

26-OHBL Is an Inactive Brassinosteroid

Bioassays with synthetic 26-OHBL demonstrate that the *CYP72B1* product is an inactive brassinosteroid in rice (Seto et al., 1999). We used the previously described observation that exogenous BL stimulates hypocotyl elongation of light-grown seedlings (Clouse et al., 1996) to determine the activity of 26-OHBL in Arabidopsis (Fig. 2). Ws-2, *cyp72b1-1*, and *cyp72b1-ox8* seedlings were unresponsive to 26-OHBL compared with BL. The *cyp72b1-ox7* and *cyp72b1-ox9* lines were used as controls to show that unresponsiveness to 26-OHBL by *cyp72b1-ox8* was not specific to the nature of the T-DNA insertion. Null mutations in *DET2* (e.g. *det2-1*) confer hypocotyls that are hyper-sensitive to exogenous BL. We have confirmed this and have demonstrated that *det2-1* seedlings are unresponsive to 26-OHBL (Fig. 2). In addition, the severe dwarfism phenotype of *det2-1* adults was rescued back to the wild-type phenotype by exogenous BL, yet this mutant was unresponsive to 26-OHBL (data not shown). That the *cyp72b1-1* null mutant is more responsive and the *cyp72b1-ox8* line is less responsive to exogenous BL compared with Ws-2 further supports the model that CYP72B1 is a BL-inactivating enzyme. Together, these results demonstrate that 26-OHBL is an inactive brassinosteroid and provide physiological and genetic evidence that conversion of BL to 26-OHBL by CYP72B1 is a steroid-hormone inactivation mechanism in Arabidopsis.

CYP72B1 Null and Overexpressing Lines Have Altered Brassinosteroid and Light Responsiveness

RNA null and overexpression lines were used to test the hypothesis that *CYP72B1* is involved in photomorphogenesis, and more specifically, deetiolation (transition from growth in the dark to growth in the light; Fig. 3A). Hypocotyl elongation inhibition in response to light is well characterized and often used as a phenotypic marker for deetiolation. We first assayed the seedling hypocotyl length of Ws-2 and the *cyp72b1-1* null mutant in

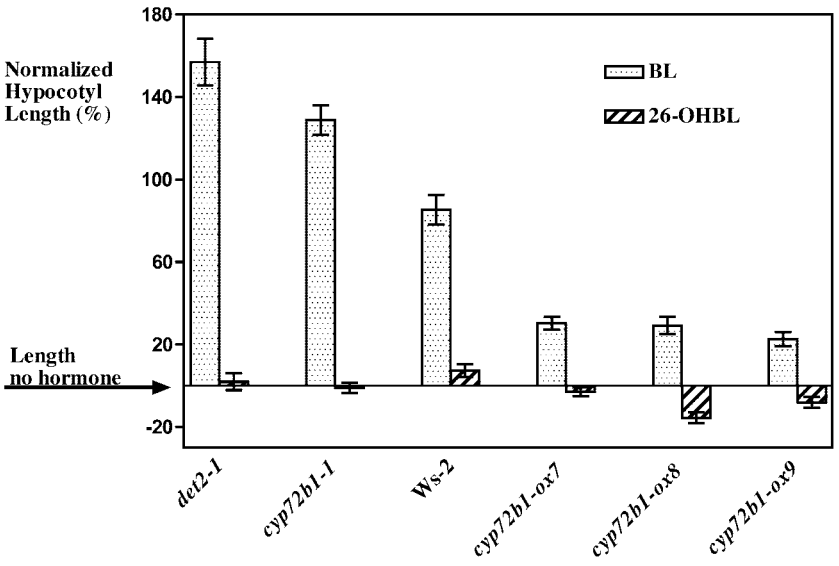


Figure 2. Biological activity of BL and 26-OHBL in Arabidopsis seedlings. Seedlings were grown for 4 d in the presence of either BL (100 nM) or 26-OHBL (100 nM) in 130 μmol m^{-2} s^{-1} white light. Hypocotyl lengths were then normalized to seedlings grown in the absence of hormone and expressed as a percentage change. Each bar represents approximately 100 seedlings from three independent replicates. Error bars represent the SE after each seedling was normalized to the average hypocotyl length of the same genotype grown in the absence of hormone.

response to increasing concentrations of exogenous BL when grown in the dark or bright-white light (Fig. 3B). There was essentially no difference between Ws-2 and *cyp72b1-1* in response to BL in the dark. The only exception was a slightly shorter *cyp72b1-1* hypocotyl at a concentration of 10 nM. In contrast, *cyp72b1-1* was slightly more responsive to increasing concentrations of exogenous BL when grown in white light (Fig. 3B). This light-dependent phenotype suggested that *CYP72B1* is involved in de-etiolation.

To further test the hypothesis that BL antagonizes de-etiolation in Arabidopsis, we assayed hypocotyl growth inhibition for Ws-2 seedlings grown at various combinations of white-light fluence rates and BL concentrations (Fig. 3C, Ws-2). The combination with the greatest response (i.e. shortest hypocotyls) had the highest white-light fluence rate and the lowest concentration of BL. In contrast, the combination with the least response had the lowest white-light fluence rate and the highest concentration of BL. A

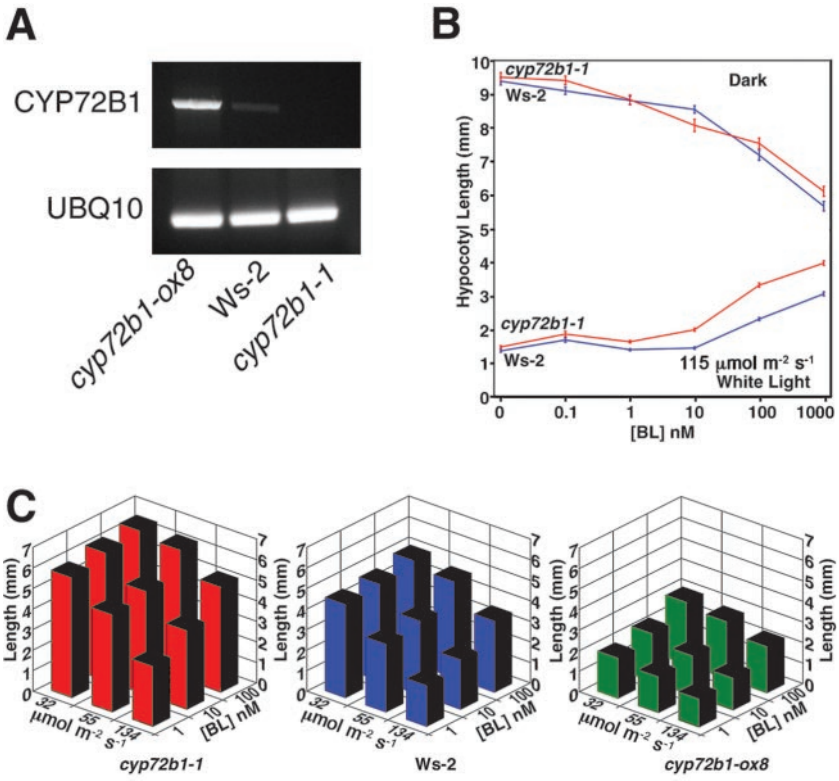
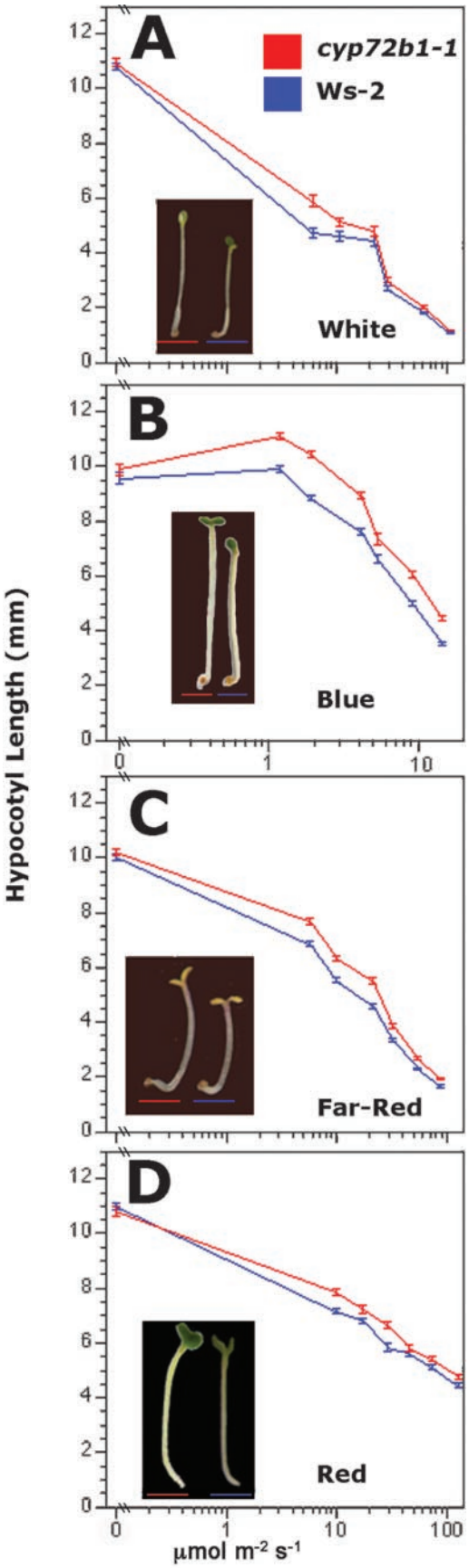


Figure 3. Physiological and genetic analysis of *CYP72B1* involvement in BL responsiveness and photomorphogenesis in white light. A, RT-PCR analysis of *CYP72B1* transcript abundance in the *CYP72B1* overexpressor (*cyp72b1-ox8*), the wild type (Ws-2), and a null allele (*cyp72b1-1*). B, Hypocotyl responsiveness to increasing concentrations of exogenous BL of 4-d-old *cyp72b1-1* and Ws-2 seedlings in the dark or 115 μmol m^{-2} s^{-1} white light. Each point represents approximately 100 seedlings combined from three independent replicates. The Ws-2 and *cyp72b1-1* means were not significantly different ($P > 0.05$) when grown in the dark under all conditions tested. The means were significantly different ($P < 0.05$) in white light at [BL] of 1 nM or greater. Error bars represent the SE. C, Hypocotyl responsiveness of 4-d-old *cyp72b1-1*, Ws-2, and *cyp72b1-ox8* seedlings at nine combinations of varying white-light fluence rate and BL concentration. Each column, representing approximately 100 seedlings combined from three replicates, is significantly different ($P < 0.05$) from its neighboring column (i.e. comparing column pairs where either light intensity or BL concentration has been changed, but not both at once) and from corresponding columns in the other genotypes.



gradation between these two extremes was produced based on the combination of the relative amounts of light and BL. These data demonstrated that light and BL are antagonistic with respect to hypocotyl growth and suggested that active brassinosteroids have a negative regulatory role in Arabidopsis de-etiolation.

If active brassinosteroids are negative regulators of de-etiolation, then brassinosteroid inactivation should act as a positive modulator of de-etiolation via the removal of such a negative regulator. The *cyp72b1-1* null mutant and the *cyp72b1-ox8* line were used to test this hypothesis (Fig. 3). Loss of *CYP72B1* expression resulted in reduced responsiveness to light (removal of a positive modulator) and increased responsiveness to BL (removal of an inactivating enzyme) at every condition tested, which resulted in a graph with the same basic pattern as Ws-2, but raised (Fig. 3C, *cyp72b1-1*). Analysis of the *cyp72b1-ox8* line showed the opposite response (Fig. 3C, *cyp72b1-ox8*). These results are consistent with a model in which *CYP72B1* is modulating de-etiolation via BL inactivation.

To begin to examine the role of CYP72B1 in the various photoreceptor response pathways, fluence rate-response analysis of *cyp72b1-1* was compared with Ws-2 in white and monochromatic light (Fig. 4). Arabidopsis uses five red/far-red absorbing photoreceptors (phytochromes A–E) and two blue/UVA photoreceptors (cryptochromes1 and 2). Reduction in responsiveness of a null mutant to white light indicates a genetic role in light responsiveness, whereas reduction in responsiveness to a particular color of light is a first step in examining a genetic role in a specific photoreceptor response pathway. All conditions resulted in no difference in the dark and a slightly taller hypocotyl in low fluence rate conditions (Fig. 4). This growth response to light is similar to the previously described *CYP72B1* antisense lines (Neff et al., 1999) and is also conferred in three additional null alleles; *cyp72b1-2*, *cyp72b1-3* and *cyp72b1-4* (data not shown). These results suggested that *CYP72B1* plays a role in multiple photoreceptor pathways.

The *CYP72B1* null and overexpression lines were assayed for responsiveness to exogenous BL at a single concentration (10 nM) and to light at a single fluence rate (Fig. 5). In the dark without BL supplementation, *cyp72b1-1* and Ws-2 were the same height, but in each light condition except blue, *cyp72b1-1* was significantly taller. In far-red light without BL supplementation, *cyp72b1-1* was 30% taller than Ws-2. In far-red light with BL supplementation, *cyp72b1-1* was 55% taller than Ws-2. A similar change in response to

Figure 4. Physiological and genetic analysis of the involvement of *CYP72B1* in light responsiveness. Hypocotyl lengths are of 4-d-old *cyp72b1-1* and Ws-2 seedlings grown in increasing fluence rates of white (A), blue (B), far-red (C), and red (D) light. Error bars represent the SE.

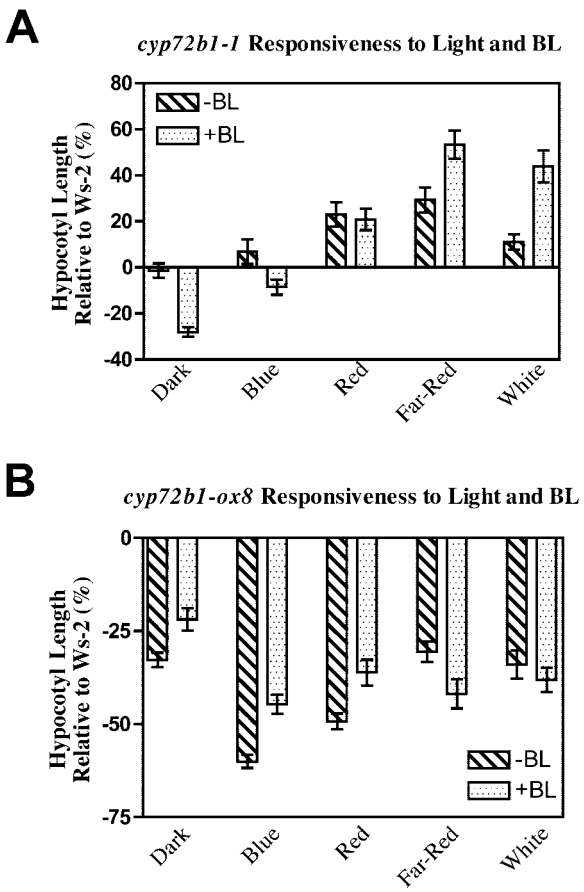


Figure 5. Physiological and genetic analysis of the involvement of *CYP72B1* in BL responsiveness and photomorphogenesis in varying light conditions. Hypocotyl lengths are of 4-d-old seedlings. Hypocotyl lengths of *cyp72b1-1* (A) or *cyp72b1-ox8* (B) normalized to Ws-2 hypocotyl lengths under the same conditions and expressed as a percentage change. Seedlings were grown in the dark, 27 μmol m^{-2} s^{-1} white, 5 μmol m^{-2} s^{-1} red, 3 μmol m^{-2} s^{-1} blue, or 30 μmol m^{-2} s^{-1} far-red light with or without BL supplementation and without Suc supplementation to the medium. Error bars represent the SE after each seedling was normalized to the average wild-type hypocotyl length. $n = 36$ seedlings per treatment.

exogenous BL was seen in white light, but not in blue or red light, which suggests that far-red light may be important to the role of CYP72B1 in photomorphogenesis (Fig. 5A).

Unlike *cyp72b1-1*, *cyp72b1-ox8* was shorter (33%) than Ws-2 in the dark without BL supplementation, and the difference was smaller on BL-containing plates (21%). This pattern of response to BL in the dark was also seen in blue- and red-light conditions (Fig. 5B). In far-red light, *cyp72b1-ox8* was 31% shorter than Ws-2 when the medium was not supplemented with BL, and the difference was larger on BL-containing plates (45%). The results from analysis of a *CYP72B1* overexpression line are therefore consistent with the results from a *CYP72B1* null line. In this assay, loss of CYP72B1 caused an apparent increase in BL sensitivity in far-red light and overexpression of CYP72B1 had the opposite result.

CYP72B1 Transcript Accumulation Is Light-Regulated

Arabidopsis seedlings were assayed for differential *CYP72B1* RNA transcript accumulation by real time reverse transcriptase (RT)-PCR (Fig. 6). *CYP72B1* and internal reference RNA (*UBQ10*) were accurately and reproducibly measured from a 2-fold serial dilution of total cDNA by this method (Fig. 6, A and B). The ratio of *CYP72B1* to *UBQ10* RNA level was then compared between various light conditions and organs (Fig. 6, C and D).

After 1 h of exposure to far-red light, there was no detectable change in *CYP72B1* transcript accumulation (Fig. 6C). In contrast, 1 h of exposure to red, blue, and white light resulted in an approximate 4-fold reduction. The presence of a single null allele of *PHYA*, *PHYB*, or *CRY1* was unable to prevent the reduction of *CYP72B1* transcript accumulation in white light (Fig. 6C). This suggests that multiple photoreceptor pathways interact to modulate *CYP72B1* transcript accumulation.

After 1 h of exposure to white light, the level of *CYP72B1* transcript in the root was unchanged (Fig. 6D). The apex and the hypocotyl, on the other hand, showed a decrease in *CYP72B1* RNA abundance similar to whole seedlings, although the hypocotyl had more *CYP72B1* RNA in both conditions.

CYP72B1 Protein Accumulation Is Light-Regulated

The entire *CYP72B1* gene from 1.5-kb upstream of the start codon to the last base before the stop codon was cloned in frame to the *uidA* gene, which encodes the β-glucuronidase (GUS) enzyme. Adult *cyp72b1-1* plants were transformed with this construct, and 30 independent single-insert (locus/unknown copy number) lines were identified. GUS assays were performed on three independent lines, which ranged from weak to strong brassinosteroid-deficient phenotypes. Seedlings were grown in continuous dark or for 3 d followed by illumination for 24 h in red, blue, far-red, or white light. GUS analysis was then performed as described in "Materials and Methods," and each line gave similar results (Fig. 7).

In all light conditions, there was an obvious GUS stain in the root at the root/hypocotyl transition zone (Fig. 7, F–J). In far-red light, there was a clear GUS stain in the elongation zone of the hypocotyl just below and often including the apical hook (Fig. 7B). CYP72B1 protein accumulation appeared unchanged between dark, red-, blue-, and white-light conditions (Fig. 7, A and C–E).

DISCUSSION

Biochemistry of CYP72B1

A previous genetic characterization using overexpression and antisense lines suggested that the CYP72B1 enzyme inactivates brassinosteroids (Neff

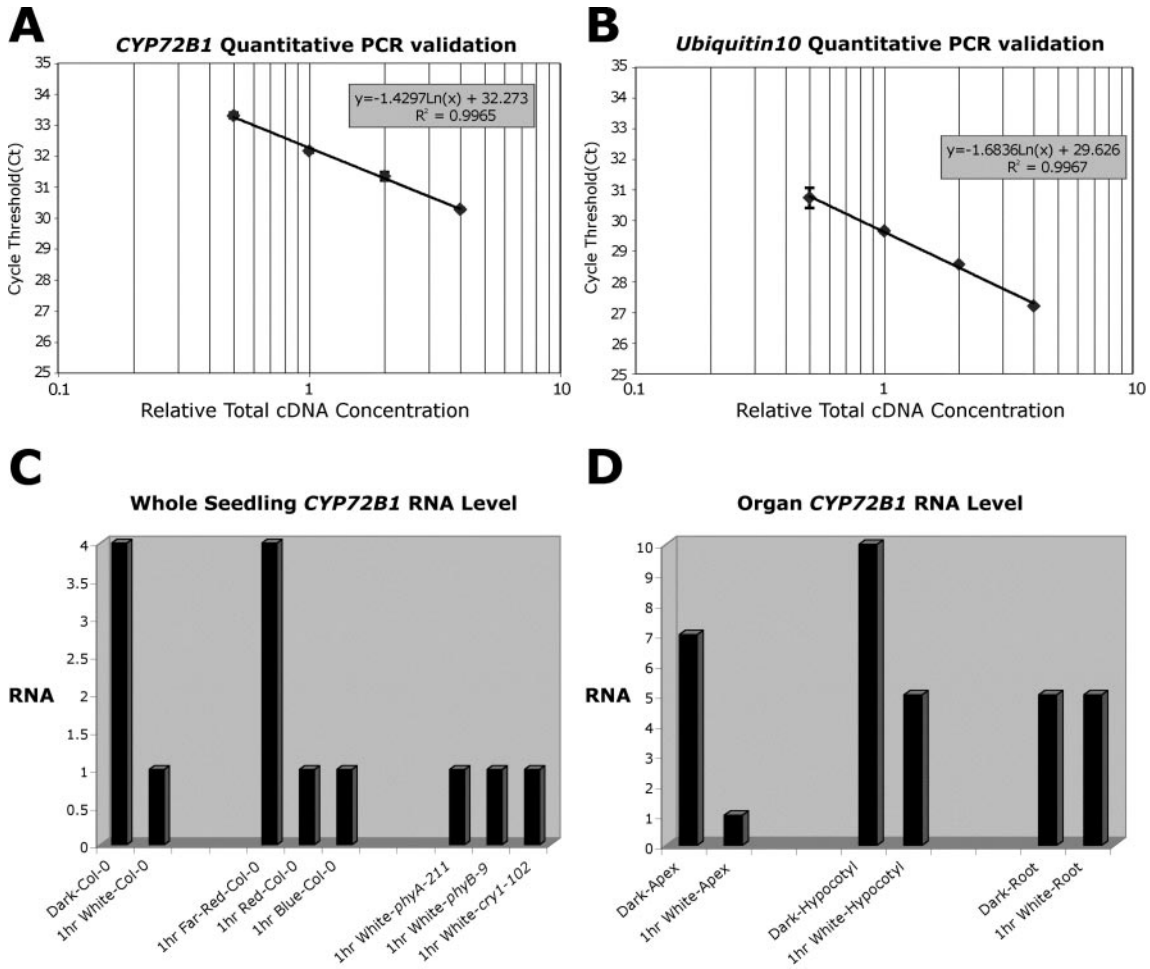


Figure 6. Organ specificity and light regulation of *CYP72B1* transcript accumulation. *CYP72B1* (A) and internal reference RNA (B; *UBQ10*) were accurately and reproducible measured from a 2-fold serial dilution of total cDNA by real time RT-PCR using light upon extension fluorescent primers. RNA abundance is measured as the PCR cycle that generates a level of fluoresce above background or cycle threshold (Ct). Error bars represent the SE. C, Whole-seedling *CYP72B1* RNA abundance of 4-d-old Col-0 seedlings grown in the dark or shifted to white, red, blue, or far-red light for 1 h and of seedlings with a null allele of *PHYA* (*phyA-211*), *PHYB* (*phyB-9*), or *CRY1* (*cry1-102*) after a 1-h shift to white light. D, Level of *CYP72B1* RNA abundance in the apex, hypocotyl, and root of dark-grown seedlings and seedlings shifted to white light for 1 h. RNA abundance in C and D is reported as the ratio of CYP72B1 to UBQ10 RNA as determined by the Ct and expressed in relative units.

et al., 1999). In this study, we demonstrated that CYP72B1 expressed in yeast can convert BL as well as CS to the 26-hydroxy derivatives (Table I). These data confirm the biochemistry of the CYP72B1 enzyme and suggest that CYP72B1 can have broad substrate specificity for active brassinosteroids. This broad substrate specificity is analogous to the Arabidopsis DWARF/CYP85 enzyme, a CYP450 involved in brassinosteroid biosynthesis (Shimada et al., 2001).

Furthermore, we showed that feeding brassinosteroids to the *cyp72b1-1* null mutant resulted in relatively less accumulation of the 26-hydroxy derivatives, whereas a transgenic line overexpressing *CYP72B1* resulted in more (Table I). The detection of a small amount of 26-OHBL in the *cyp72b1-1* null mutant suggests the presence of a redundant CYP450. CYP72C1 is a candidate for providing the redundant 26-OHBL activity. The dwarf phenotypes of *chibi2*, an activation-tagged Arabidopsis mutant with high *CYP72C1* transcript levels, are similar to those of *bas1*-D (see Bishop and Yokota, 2001).

Relative to wild-type controls, bioassays with synthetic 26-OHBL supplemented to the growth medium also supported the notion that CYP72B1 enzymatic activity results in an inactive brassinosteroid (Fig. 2). Bioassays with synthetic 28-OHBL resulted in typical brassinosteroid responses, which demonstrate that addition of one hydroxyl group to the BL side chain does not impair uptake of the resulting molecule (Seto et al., 1999), suggesting that 26-OHBL can be taken up by plants.

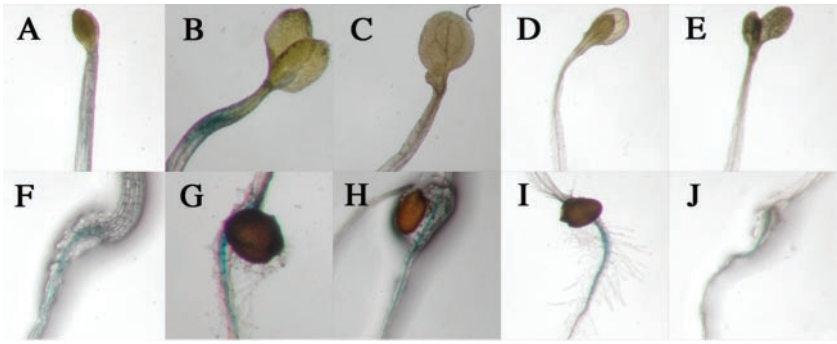


Figure 7. Organ specificity and light regulation of *CYP72B1* protein accumulation. Photographs of 4-d-old transgenic seedlings containing the GUS gene fused to the N terminus of the CYP72B1 full gene and promoter. The elongation zone of the hypocotyl just below and including the apical hook (A–E) and the root/hypocotyl transition zone (F–J) are pictured. In each light condition, the tissues photographed are from the same seedling. Seedlings were grown in the dark (A and F) and were transferred for 24 h to far-red (B and G), blue (C and H), red (D and I), or white (E and J) light. Seedlings that were not transformed with the GUS reporter fusion did not show GUS staining (not pictured).

Taken together, these results support a mechanism of brassinosteroid hormone inactivation in plants, which is analogous to the inactivation of the insect molting hormone ecdysone. Namely, the CYP450-mediated hydroxylation of an active hormone at the carbon-26 position resulting in an inactive congener. Interestingly, ecdysone is the non-plant steroid that is structurally the most similar to BL (Voigt et al., 2001).

Involvement of CYP72B1 in Photomorphogenesis

We also tested the hypothesis that *CYP72B1* is involved in photomorphogenesis. This developmental pathway can be assayed by measuring the response of Arabidopsis hypocotyl elongation to white or monochromatic light. A simple genetic test is to determine whether a line that is defective in the gene of interest is also less responsive to these conditions. The *cyp72b1-1* hypocotyls were less responsive to all light conditions tested, suggesting that *CYP72B1*-mediated inactivation of brassinosteroids is involved in each of the pathways that make up photomorphogenesis (Fig. 4). This simple test does not rule out the possibility that *CYP72B1*/brassinosteroids act on hypocotyl elongation in a pathway that is parallel to light inhibition of hypocotyl elongation. For example, an attractive hypothesis is that light simultaneously activates growth-inhibiting and -promoting pathways so that the rate of hypocotyl elongation in any condition is the resultant or balance between two opposing influences of light (Parks et al., 2001). A light-dependent reduction in the deactivation of brassinosteroids could be part of the promoting pathway that works against, but independently of, the inhibitory action of the photoreceptors in wavelengths other than far-red. That the *cyp72b1-1* mutant has normal hypocotyl growth in the dark suggests that hypocotyl elongation is not simply impaired by this mutation. Therefore, the light-dependent hypocotyl phenotype conferred in the *cyp72b1-1* null mutant suggests that *CYP72B1* is involved in photomorphogenesis and provides genetic evidence of cross-talk between photomorphogenesis and regulation of active brassinosteroid levels.

Mutants defective in brassinosteroid biosynthesis (*det2/cpd*) or perception (*bri1*) are short in the dark as well as in the light. This demonstrates that brassinosteroids are required for the hypocotyl to elongate. In fact, addition of BL to the growth medium has a stimulatory affect on hypocotyl elongation of light-grown seedlings. A seemingly contradictory phenomenon is the ability of exogenous BL to inhibit hypocotyl elongation in the dark at concentrations that have a stimulatory effect in the light (Fig. 3B). This suggests that the BL response is nearly saturated in the dark, and as a consequence, increases in concentration of this growth-promoting hormone act to inhibit or "poison" the response. This response dichotomy may be due to differential BL sensitivity between the two environments.

An analogous mechanism is the dichotomy of response to auxin between the shoot and the root in response to gravity (Ottenschlager et al., 2003). Auxin accumulates in the lower portion of the root to inhibit cell elongation and affect downward bending, whereas auxin accumulation in the shoot stimulates cell elongation to cause upward bending. On the basis of this analogy, we propose a model in which the dark-grown seedling is more sensitive to brassinosteroids than the light-grown seedling. Upon illumination, the seedling could become less sensitive to brassinosteroids, allowing a rapid shutting off of the growth promoting effect in the hypocotyl. The level of brassinosteroids could also be decreased to complement the change in sensitivity. No change or even an increase in the level of brassinosteroids would also be compatible with a change in sensitivity model.

Loss of CYP72B1 had only a minor effect on the BL dose response of dark-grown seedlings. At 10 nM BL, the dark-grown seedlings are shorter/more-

responsive in the *cyp72b1-1* background compared with Ws-2 (Figs. 3B and 5A). Although in the absence of exogenous BL, *cyp72b1-1* is the same height as Ws-2 in the dark. This suggests that *CYP72B1* does not play a role in skotomorphogenesis but may play a role in the inactivation of exogenous BL in the dark. Recent microarray data have shown that *CYP72B1* transcript accumulation increases in response to exogenous BL (Goda et al., 2002), possibly as a feedback mechanism for controlling levels of active brassinosteroids during plant development.

Effect of Far-Red Light on CYP72B1 Protein and Transcript Accumulation

We present data that investigate the interaction between light and *CYP72B1* at the genetic, RNA, and protein level. In all cases the connection was strongest with far-red light. Furthermore, double mutant analysis of *bas1*-D and a phytochrome A null allele (*phyA-201*) demonstrated that the increased responsiveness of the overexpressor to far-red light is dependent on the photoreceptor phyA (Neff et al., 1999). Taken together, the data suggest that brassinosteroid catabolism is more important in far-red-light-mediated photomorphogenesis compared with the red- and blue-light-mediated pathways. A reduction in brassinosteroid sensitivity in conjunction with a decrease in brassinosteroid levels in far-red light may explain why seedling inhibition of elongation is strongest in far-red light. Mutants affected in brassinosteroid biosynthesis also have reduced high-irradiance and low-fluence responses, while having an enhanced very-low-fluence response. These results were interpreted to suggest that brassinosteroids are involved in the fine-tuning of phytochrome signaling (Luccioni et al., 2002). A careful fluence rate-response analysis of double mutants of *cyp72b1-1* and null alleles of the various photoreceptors will be valuable to further characterize the role of *CYP72B1* in the far-red-light response pathway.

Exogenous BL can also elicit increases in *CYP72B1* RNA abundance (Goda et al., 2002). Conclusions about the levels of active brassinosteroids based on the presence of the brassinosteroid-inactivating enzyme CYP72B1 must therefore be made with caution. One specific way to interpret the increase in *CYP72B1* RNA in response to exogenous BL is that CYP72B1 is made where and when it is needed. For example, it is reasonable that CYP72B1 would be needed in response to sudden increases in BL to reduce the pool to a level that the tissue can again respond to. It is also reasonable that CYP72B1 would be needed in response to an environmental cue, such as light, to reduce the steady-state brassinosteroid level to reduce the rate of hypocotyl elongation.

CONCLUSIONS

The original hypothesis that CYP72B1 is a BL carbon-26 hydroxylase has been confirmed using yeast and Arabidopsis with altered *CYP72B1* expression and has been extended to include carbon-26 hydroxylation of CS. One of the products of this enzymes activity, 26-OHBL, has also been shown to be a functionally inactive brassinosteroid during seedling development.

The brassinosteroid sensitivity phenotypes in far-red light by the *CYP72B1* null and overexpression lines, coupled with the observation that *CYP72B1* transcript levels are not decreased and the protein accumulation is increased by brief exposure to far-red light, suggests that far-red light modulates brassinosteroid levels in part by the action of CYP72B1. If far-red light also modulates seedling sensitivity to brassinosteroids, then the effect of CYP72B1, and brassinosteroid inactivation in general may be amplified. The lack of blue- and red-light brassinosteroid sensitivity phenotypes in the CYP72B1 null, coupled with the lack of detectable GUS protein reporter and decreased RNA in theses conditions, suggests that red and blue light may provide only weak modulation of brassinosteroid levels through the action of CYP72B1.

MATERIALS AND METHODS

Plant Material

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes. Arabidopsis ecotype Ws-2 was used as the wild-type starting material for the generation of a null allele of *CYP72B1* (*cyp72b1-1*) and the *CYP72B1* overexpression line (*cyp72b1-ox8*). A null allele line of *DET2* (*det2-1*), ecotype Col-0, was used for analysis of 26-OHBL activity. The photoreceptor null alleles used in the quantitative RT-PCR are in the Col-0 ecotype: *CRY1* (*cry1-102*), *PHYA* (*phyA-211*), and *PHYB* (*phyB-9*).

The T-DNA insertion line (*cyp72b1-1*) came from the Wisconsin Knockout Facility (Madison; http://www.biotech.wisc.edu/Arabidopsis/) and was identified at the University of Arizona (Tucson; http://Ag.Arizona.Edu/p450/). The original *cyp72b1-1* line was back-crossed to Ws-2, and F_2 seeds were collected from kanamycin-resistant (Kan^r) F_1 plants. Each F_2 population segregated approximately 15 Kan^r to 1 kanamycin-sensitive (Kan^s) seedling, indicating that the original line contains two unlinked loci of T-DNA insertion. F_3 seeds from Kan^r F_2 parents were assayed for Kan^r, and those with a 3:1 ratio (Kan^r:Kan^s) were analyzed by PCR for the presence of the T-DNA in the *CYP72B1* open reading frame. All F_4 seed populations from an F_3 line containing the T-DNA in *CYP72B1* were either homozygous for Kan^r or segregated 3:1 (Kan^r:Kan^s). PCR analysis showed that those populations homozygous for Kan^r were also homozygous for the T-DNA insertion in *CYP72B1*. Sequencing of the T-DNA insertion site showed the insertion to occur within the second exon, at 1,412 bp downstream of the ATG.

For this study, pMN17 (*CYP72B1* gene and promoter driven by 4 × 35S enhancer elements) was randomly inserted into the genome of Arabidopsis ecotype Ws-2 via *Agrobacterium tumefaciens*-mediated transformation to create a *CYP72B1* overexpression series. Plasmid pMN17 is described by Neff et al. (1999). Forty gentamycin-resistant T_1 seedlings were randomly selected and grown to maturity under long-day/greenhouse conditions. Twenty-four T_1 plants were discarded due to low seed set, and the remaining 16 T_1 plants (several thousand seeds each) were designated *cyp72b1-ox1* through *cyp72b1-ox16*. All 16 lines likely contain a single locus of T-DNA insertion as inferred by a 3:1 ratio of gentamycin-resistant to -sensitive T_2 seeds with one or more copies per locus.

Fifteen T_2 seedlings from each of the 16 lines were transplanted to soil and grew to maturity in greenhouse/long-day conditions. Each T_2 plant was harvested individually, and an aliquot of each of the T_3 seed pools was surface sterilized and sprinkle plated onto gentamycin-containing plates as above. Each T_3 line was scored for the ratio of gentamycin resistance versus sensitivity and uniformity of seedling phenotype. Eight lines with good seed set and greatest degree of seedling uniformity were kept. All homozygous resistant T_3 seedlings from each of the eight remaining lines were transferred from plates to soil (3-inch pots, four seedlings per pot). The *cyp72b1-ox8* line is a weak *CYP72B1* overexpressor (based on severity of the adult dwarfism phenotype), having a uniform phenotype between siblings and good seed set. The transgenic lines *cyp72b1-ox7* and *cyp72b1-ox9* also have a uniform phenotype and good seed set, but they both have a greater degree of dwarfism (marker for high expression).

Surface Sterilize Seeds

Seeds were surface sterilized by 5 min of agitation in 70% (v/v) ethanol with 0.05% (v/v) Triton X-100, followed by 5 min of agitation in 95% (v/v) ethanol with 0.05% (v/v) Triton X-100, and 5 min of agitation in 95% (v/v) ethanol. A disposable Pasteur pipette was used to transfer the seed/ethanol solution to a sterilized 90-mm filter paper placed inside a sterile petri dish. Seeds were allowed to air dry in a sterile laminar-flow hood and then sown immediately onto growth medium or sealed in the petri dish with parafilm and stored at 4°C.

Medium and Seed Sowing

Surface-sterilized seeds were sown onto 90-mm square petri dishes containing 1% (w/v) phytagel (Sigma, St. Louis), 1.5% (w/v) Suc, and one-half Murashige and Skoog salts (catalog no. 11118-023, Invitrogen, Carlsbad, CA) in a sterile laminar flow hood. Seeds were sown individually and spaced approximately 10 mm apart in a triangular pattern using a sterile 200-μL pipette tip. Sown petri dishes were then wrapped in aluminum foil and incubated upside down in a 4°C cold room for 5 d to stratify seeds for uniform germination. Hormone plates contained BL dissolved in 95% (v/v) ethanol (CIDtech Research Inc., Missisuaga, ON, Canada), 26-OHBL dissolved in 95% (v/v) ethanol (see Neff et al., 1999), or no hormone with the appropriate amount of 95% (v/v) ethanol. All brassinosteroids or controls were added to autoclaved medium that had been cooled to 65°C just before pouring.

Growth Conditions

After 5 d of incubation at 4°C, plates were unwrapped and incubated for 30 min in a growth chamber containing red-light-emitting diodes (model E-30B, Percival Scientific, Perry, IA) to induce uniform germination. Plates were then incubated in the dark for 23.5 h and then incubated in a similar growth chamber, but with white light, for 72 h. All chamber temperatures were at 24°C. White light of 130 μmol m^{-2} s^{-1} (measured by a LI-250 Light Meter [LI-COR, Lincoln, NE]) was derived from eight fluorescent tubes (F17T8/TL741 17WATT) from Philips (Eindhoven, The Netherlands) and two incandescent tubes (PC: 13487 25T10/F CD 25WATTS 120 V) from GE (Fairfield, CT). One to six layers of gray, fiberglass window screening were used to vary the fluence rate.

Seedling Measurements

After 4 d of growth, plates were moved to 4°C cold room for storage until measured. Seedlings were gently lifted with forceps from plates onto acetate sheets and digitized with a flat-bed scanner at a resolution of 1,200 dpi. Seedling scans were measured using ImageJ 1.29j software (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/java1.3.1).

Construction of pED10 for Translational Fusion of CYP72B1 to GUS

The entire *CYP72B1* gene from just 5′ of the stop codon to 1.5 kb 5′ of the translational start codon was amplified from BAC F18A8 using the following primers: forward 5′-AGT TAC GGT CGA CAC TCT TGC CTT GGT TAG A, reverse 5′-GGA TTG CTT CTC CAT GGA ATC CTC ATG ATT GGT CA. The resulting product was then ligated into pCAMBIA1301 after digestion with the restriction endonucleases *Bam*HI and *Nco*I. Amplification and sequencing was performed as below with pED8.

GUS Assay

Whole seedlings were transferred from plates to a 1.5-mL centrifuge tube containing 1 mL of GUS staining solution (1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexyl ammonium salt, 50 mM NaHPO4, pH 7.2, and 0.5% [v/v] Triton X-100) and incubated at 37°C for 48 h. 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexyl ammonium salt was purchased from Research Products International (Mt. Prospect, IL). Seedlings were photographed with a stereoscopic zoom microscope (SMZ800, Nikon, Tokyo) with Nikon coolpix990 digital camera immediately or after a 1-h incubation in 75% (v/v) ethanol (to remove chlorophyll).

Construction of pED8 for Overexpression of *CYP72B1* in Yeast

The coding region of *CYP72B1* cDNA was cloned into the pYeDP60 yeast expression vector (Pompon et al., 1996). The cDNA was amplified by PCR with the following forward and reverse primers, respectively: YEF1 5′-GGT AGG ATC CAT GGA GGA AGA AAG TAG CAG and YER1 5′-GGT TCA ATT GTC AAT CCT CAT GAT TGG TC. The resulting product was digested with the restriction endonucleases *Bam*H I and *Mfe* I, which were introduced into the PCR product via the primers (see underlined sequences). Amplification was performed with the Advantage-HF2 PCR kit (BD Biosciences Clontech, Palo Alto, CA) following the manufacturer's instructions. PCR fidelity was confirmed by sequencing the entire insert with at least 2-fold coverage.

Yeast Functional Assay

Yeast expression of the *CYP72B1* gene was performed as described previously (Bishop et al., 1999; Shimada et al., 2001). An ethanol solution (5 μg 5 μL^{-1}) of BL, [$^2H_6$]BL, CS, or [$^2H_6$]CS was added to a culture as a substrate for CYP72B1 protein. After approximately 16 h of incubation, the culture was extracted with ethyl acetate. The ethyl acetate-soluble fraction was purified with a silica gel cartridge (Sep-Pak Vac 2g; Waters, Milford, MA), which was eluted with 40 mL of chloroform, 2% (v/v) methanol in chloroform, and 20% (v/v) methanol in chloroform. The 20% (v/v) methanol fraction was purified by an ODS cartridge column, and then purified by HPLC on a 150- × 4.6-mm Senshu Pak ODS-1151-D column (Senshu Scientific, Tokyo) using 45% (v/v) acetonitrile as the mobile phase at a flow rate of 1.0 mL min^{-1}. Under these conditions, 26-OHBL and 26-OHCS were detected in the range of 2 to 4 min; BL was detected in the fraction of tR: 6 to 10 min; and CS was detected in the faction of tR: 10 to 15 min. BL and CS fractions were treated with pyridine containing methaneboronic acid (40 μg 20 μL^{-1}) at 80°C for 30 min. 26-OHBL and 26-OHCS fractions were treated with pyridine containing methaneboronic acid (20 μg 10 μL^{-1}) at 80°C for 30 min and then with 10 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide at 80°C for 30 min. The derivatized fractions were analyzed by GC-MS.

GC-MS Analysis

GC-MS analysis was carried out on a mass spectrometer (JMS-AM SUN200, JEOL, Tokyo) connected to a gas chromatograph (6890A, Agilent Technologies, Wilmington, DE) with a capillary column DB-5 (0.25 mm × 15 m, 0.25-μm film thickness; J&W Scientific, Folsom, CA). Analysis was conducted under the following conditions: ionization, EI (70 eV); carrier gas, helium at a flow rate of 1 mL min^{-1}; injection temperature, 280°C; column temperature, 80°C for 1 min, elevated to 320°C at 30°C min^{-1}, and then maintained at 320°C.

Metabolism of BL, [$^2H_6$]BL, CS, And [$^2H_6$]CS in Arabidopsis Seedlings

For metabolic experiments, Arabidopsis seeds were surface sterilized and placed on petri plates containing one-half-strength Murashige and Skoog

medium supplemented with 1% (w/v) Suc and 0.8% (w/v) agar at 22°C in the light. Seven days after sowing, seedlings (Ws-2, *cyp72b1-1*: 15 seedlings, and *cyp72b1-ox8*: 30 seedlings) were transferred to 200-mL flasks containing 30 mL of one-half-strength Murashige and Skoog medium supplemented with 1% (w/v) Suc. The seedlings were grown at 22°C in the light on a shaker (120 rpm). Nine days after transfer, an ethanol solution (10 μg 10 μL^{-1}) of BL, CS, [$^2H_6$]-BL, or [$^2H_6$]-CS was added to each flask. The seedlings were allowed to grow for another day under the same growth conditions and then extracted with methanol. The methanol extract was partitioned between ethyl acetate and water. The ethyl acetate-soluble fraction was purified and analyzed by the methods described above.

Quantitative RT-PCR

CYP72B1 transcript accumulation was measured as the ratio of *CYP72B1* RNA level to *ubiquitin10* (*UBQ10*) RNA level (internal reference). Each RNA level was measured as the cycle threshold above background fluorescence using a Smart Cycler real time PCR machine from Cepheid (Sunnyvale, CA). The *CYP72B1* PCR product is 6-carboxy-fluorescein labeled, and *UBQ10* is 6-carboxy-4′,5′-dichloro-2′,7′-dimethyoxy-fluoresein labeled due to incorporation of light upon extension primers from Invitrogen (Carlsbad, CA). The *CYP72B1* forward primer is labeled on the third base from the 3′ end: 5′-CAA CAA GCG GCG AAG GAT TTG TTG. The *CYP72B1* reverse primer is unlabeled: 5′-CAC TCC TCC ACC AAT GTC CTG AA. The *UBQ10* forward primer is labeled on the third base from the 3′ end: 5′-GTA CGT CGC AGC TTG AGG ATG GAC GTA C. The *UBQ10* reverse primer is unlabeled: 5′-ACG AAG ACG CAG GAC CAA GT. Total RNA was isolated from 18 × 4-d-old seedlings using the RNeasy Plant Mini Kit from Qiagen (Valencia, CA). Total cDNA was produced using the SuperScriptIII First-Strand Synthesis System for RT-PCR from Invitrogen of which 0.5 μL was used in a 25-μL PCR reaction using the Platinum Quantitative SuperMix-UDG from Invitrogen. *CYP72B1* PCR cycle parameters: 50°C/120 s; 95°C/120 s; 45 repeats, 95°C/15 s, 68°C/30 s. *UBQ10* PCR cycle parameters: 50°C/120 s; 95°C/120 s; 45 repeats, 95°C/60 s, 64°C/60 s.

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