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Citation for published version:

Bancos, S, Szatmári, A-M, Castle, J, Kozma-Bognár, L, Shibata, K, Yokota, T, Bishop, GJ, Nagy, F & Szekeres, M 2006, 'Diurnal regulation of the brassinosteroid-biosynthetic CPD gene in *Arabidopsis*', *Plant physiology*, vol. 141, no. 1, pp. 299-309. <https://doi.org/10.1104/pp.106.079145>

Digital Object Identifier (DOI):

[10.1104/pp.106.079145](https://doi.org/10.1104/pp.106.079145)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Plant physiology

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Diurnal Regulation of the Brassinosteroid-Biosynthetic *CPD* Gene in *Arabidopsis*^{1[W]}

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Plant steroid hormones, brassinosteroids (BRs), are essential for normal photomorphogenesis. However, the mechanism by which light controls physiological functions via BRs is not well understood. Using transgenic plants carrying promoter-luciferase reporter gene fusions, we show that in *Arabidopsis* (*Arabidopsis thaliana*) the BR-biosynthetic *CPD* and *CYP85A2* genes are under diurnal regulation. The complex diurnal expression profile of *CPD* is determined by dual, light-dependent, and circadian control. The severely decreased expression level of *CPD* in phytochrome-deficient background and the red light-specific induction in wild-type plants suggest that light regulation of *CPD* is primarily mediated by phytochrome signaling. The diurnal rhythmicity of *CPD* expression is maintained in *brassinosteroid insensitive 1* transgenic seedlings, indicating that its transcriptional control is independent of hormonal feedback regulation. Diurnal changes in the expression of *CPD* and *CYP85A2* are accompanied by changes of the endogenous BR content during the day, leading to brassinolide accumulation at the middle of the light phase. We also show that *CPD* expression is repressed in extended darkness in a BR feedback-dependent manner. In the dark the level of the bioactive hormone did not increase; therefore, our data strongly suggest that light also influences the sensitivity of plants to BRs.

Plant development is determined by complex interaction between endogenous programs and environmental signals, with light being one of the most important environmental cues. Light controls essential biological processes, such as germination, shade avoidance, de-etiolation, phototropism, chloroplast movement, stomatal opening, circadian entrainment, and flowering. The plasticity of plant responses to light is ensured by multiple photoreceptors having different wavelength specificities and sensitivities, and activat-

ing target genes through either distinct or integrated signaling mechanisms. In *Arabidopsis* (*Arabidopsis thaliana*), red/far-red signals are perceived by five phytochromes, PHYA to PHYE, whereas two types of cryptochromes and phototropins function as blue and UVA light receptors (for review, see Sullivan and Deng, 2003; Chen et al., 2004). Light regulation is crucial for coordinating the metabolic and physiological functions of plants with the alternating light and dark periods during the day. In addition to direct, light-dependent diurnal regulation of gene activities, light signals are crucial for entrainment of the endogenous circadian clock that ensures cyclic expression of circadian-controlled genes with a roughly 24-h period length, thereby contributing to the precise timing/phasing of numerous biochemical and physiological processes during the day. On the other hand, a hallmark of circadian rhythms is that they persist for several days under constant environmental conditions (McClung, 2001). The importance of these processes can be gauged by the fact that approximately 11% of the genes in *Arabidopsis* show diurnal patterns of expression and that about 25% of these are also under circadian regulation (Schaeffer et al., 2001).

Whereas the perception of light signals is an intracellular process, responses at the tissue and organ levels are mediated and coordinated through the action of phytohormones. Several lines of evidence suggest that almost all hormone groups participate in the control of photomorphogenic processes and that this involves complex cross talk between the signaling routes of the

¹ This work was supported by the Hungarian Scientific Research Fund (grant nos. T 42639 to M.S. and F 47013 to L.K.-B.), an International Joint Project grant of the Royal Society (to G.J.B. and M.S.), research grants from the Biotechnology and Biological Sciences Research Council (to G.J.B.) and the Human Frontiers Science Program (RG00162-2000 to T.Y. and G.J.B.) and a Grant-in-Aid for Scientific Research (B13460050 to T.Y.) from the Ministry of Education, Science, Sports and Culture of Japan.

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^[W] The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.106.079145.

different hormones. Light can control the effect of phytohormones by influencing their biosynthesis, metabolism, transport, and/or perception (Nemhauser and Chory, 2002).

One hormone group that has been implicated in photobiological responses is brassinosteroids (BRs). In addition to the role of these steroidal phytohormones in promoting growth, fertility, and stress resistance, they also regulate photomorphogenesis, and seedlings of some of the first characterized BR-deficient mutants were shown to have short hypocotyls, open cotyledons, and elevated expression of light-regulated nuclear genes when grown in the dark (Chory et al., 1991; Li et al., 1996; Szekeres et al., 1996). Physiological effects of BRs depend on local hormone concentration and responsiveness at the sites of action. The actual levels of bioactive BRs depend on the balance between their tightly controlled biosynthesis and inactivation (Fujioka and Yokota, 2003).

BRs are synthesized from phytosterols through multiple, mostly oxidative reactions leading to brassinolide (BL), the biologically most active BR. In vivo conversion studies revealed two alternative reaction routes, the early and late C-6 oxidation pathways that utilize 6-oxo or 6-deoxo intermediates, respectively (Choi et al., 1997). In Arabidopsis, several of the biosynthetic enzymes have been identified through the characterization of BR-deficient mutants, and those catalyzing oxidative conversions were found to be cytochrome P450 monooxygenases of the CYP85 or CYP90 families (for recent review, see Fujioka and Yokota, 2003). Relaxed substrate specificities of these P450 enzymes allow conversions along parallel routes, and recent analyses suggest that BRs are synthesized via a complex network of reactions (Fujioka et al., 2002; Shimada et al., 2003).

The severe BR-deficient phenotype caused by brassinazole, a specific inhibitor of BR biosynthesis, suggests that de novo synthesis is a key factor in determining the level of the bioactive hormone (Asami et al., 2000). Analysis of the intermediate pools in Arabidopsis, tomato (*Lycopersicon esculentum*), and rice (*Oryza sativa*) revealed conserved regulation of the BR pathway, in which C-6 oxidation, as well as steroid side chain hydroxylation at C-22 and C-23, were identified as potential rate-limiting reactions (Nomura et al., 2001). In Arabidopsis, C-6 oxidation is catalyzed by two enzymes, CYP85A1 and CYP85A2, of partially redundant functions. Under in vitro conditions, yeast-expressed CYP85A1 was shown to generate teasterone, typhasterol, and bioactive castasterone from their respective 6-deoxo precursors, whereas CYP85A2, in addition to these reactions, also catalyzed the Baeyer-Villiger oxidation of castasterone to BL (Shimada et al., 2001, 2003; Kim et al., 2005; Nomura et al., 2005). The C-22 and C-23 hydroxylation reactions were found to be mediated by DWF4/CYP90B1 and CPD/CYP90A1, respectively (Szekeres et al., 1996; Choe et al., 1998). Recent studies indicated that in Arabidopsis the expression of all BR-biosynthetic P450 genes is under

both developmental and organ-specific regulation that take place primarily at the level of transcription (Bancos et al., 2002; Shimada et al., 2003; Kim et al., 2006). This can influence the efficiency of biosynthesis because activities of certain genes encoding rate-limiting enzymes, such as CPD in Arabidopsis or DWARF/CYP85A1 and CYP85A3 in tomato, show good correlation with the accumulation of active BRs (Bancos et al., 2002; Montoya et al., 2005; Nomura et al., 2005).

Homeostatic regulation, ensuring the adjustment of optimal BR concentrations under various physiological conditions, also requires temporal or permanent inactivation of excess hormone. In Arabidopsis, mutants overexpressing the *BAS1/CYP734A1* and *CHI2/SHK1/SOB7/CYP72C1* genes were shown to reduce the level of active BRs (Neff et al., 1999; Nakamura et al., 2005; Takahashi et al., 2005). In vivo conversion assays revealed that CYP734A1 (formerly CYP72B1) converts castasterone and BL to biologically inactive C-26-hydroxylated products, while in the case of CYP72C1 inactivation of the same substrates is achieved by a different, yet unknown mechanism (Turk et al., 2003, 2005). Biosynthesis and inactivation are coordinated via BR-dependent transcriptional control of the genes involved in both processes. All BR-biosynthetic P450 genes of Arabidopsis are under negative feedback regulation by active BRs (Bancos et al., 2002; Tanaka et al., 2005), whereas of those encoding inactivating enzymes only *BAS1* was found BR inducible (Choe et al., 2001; Takahashi et al., 2005). Feedback regulation of *CPD* is abolished in the BR-insensitive *bin2* and *brassinosteroid insensitive 1-2 (bri1-2)/cbb2* mutants (Li et al., 2001; Bancos et al., 2002), indicating a key role of BR signaling in the hormonal self-regulation of BR biosynthesis and perhaps also inactivation. Recently BRASSINAZOLE RESISTANT 1 (BZR1), a nuclear component of this signaling cascade, has been identified as the transcriptional repressor that can down-regulate BR-biosynthetic genes by direct binding to their promoters (He et al., 2005).

Expression studies of the Arabidopsis *CYP85* and *CYP90* genes by reverse transcription (RT)-PCR provided valuable clues regarding the timing and spatial distribution of BR synthesis (Bancos et al., 2002; Shimada et al., 2003), but low transcript levels limited the resolution and reliability of these analyses. More precise localization of transcriptional activity could be achieved in transgenic plants carrying reporter genes fused to the *CPD*, *CYP85A1*, or *CYP85A2* promoters (Mathur et al., 1998; Castle et al., 2005), proving the efficiency of reporter-based expression analysis. Because *CPD* is more actively transcribed than other BR-biosynthetic P450 genes (Shimada et al., 2003), we generated transgenic Arabidopsis lines expressing firefly (*Photynus pyralis*) luciferase (*LUC*) under control of the *CPD* promoter in order to allow in vivo monitoring of temporal changes in promoter activity by luminescence imaging. Here, we report that expression of the *CPD:LUC* fusion follows a complex diurnal pattern that is determined by both light and circadian

regulation. Very similar diurnal cycles were observed with *LUC* driven by the promoter of *CYP85A2*, the gene required for BL synthesis. We also show that light induction of *CPD* is mediated primarily by phytochrome signaling, and that both light and circadian control of promoter activity are independent of the feedback regulation by BRs. Our analyses of the endogenous hormone content revealed BL accumulation in the light, suggesting that the level of active BRs is influenced by the diurnal expression of rate-limiting biosynthetic enzymes.

RESULTS

Diurnal Changes in the Expression of BR-Biosynthetic Genes

Fusion constructs with firefly *LUC* reporter offer a flexible, noninvasive system for studying the *in vivo* activity of BR-biosynthetic gene promoters. Initial characterization of Arabidopsis lines carrying *CPD* promoter-driven *LUC* revealed that the luminescence levels detected by a CCD camera system vary depending on the time of the day. In time-course experiments, *CPD:LUC* transgenic seedlings that were raised under alternating 12-h-light/12-h-dark photoperiods (LD) showed a characteristic diurnal pattern of *LUC* activity (Fig. 1A). Following lights-on, a sudden increase of luminescence intensity resulted in a maximum, then values gradually decreased, reaching a minimum around midday, then increased to a second maximum at lights-off. Thereafter, the detected luminescence decreased again throughout the night until a second minimum at the end of the dark phase.

To see if other BR-biosynthetic genes can show similar diurnal changes of activity, we also generated *LUC* fusion with the promoter of *CYP85A2*. This gene encodes a BR C-6 oxidase catalyzing the last conversion steps of BL synthesis in Arabidopsis (Kim et al., 2005; Nomura et al., 2005), and its organ-specific expression pattern has been shown to be similar to that of *CPD* (Bancos et al., 2002; Castle et al., 2005). Although the luminescence emitted by *CYP85A2:LUC* transgenic seedlings was about 10-fold lower than that of the *CPD:LUC*-carrying ones, it also exhibited a diurnal profile with maxima and minima at similar time points as seen in the case of *CPD* (Fig. 1B). But, compared to *CPD*, *CYP85A2* promoter activity showed a broader morning peak, and its daily changes had higher amplitude.

To ascertain that the expression data obtained with the *CPD:LUC* construct faithfully reflect the activity of the *CPD* promoter, in 1-week-old seedlings we determined the steady-state levels of the *CPD* mRNA during the LD cycle using semiquantitative RT-PCR assay (Fig. 1C). The amount of the transcript was found lowest before lights-on and highest before lights-off, whereas intermediate values were observed at midday and in the middle of the night. These data are in

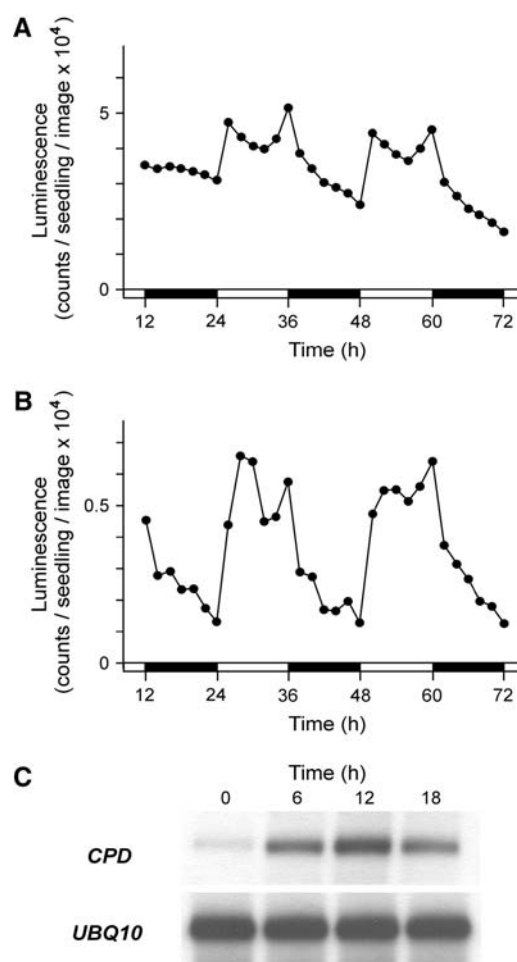


Figure 1. Diurnal expression of the BR-biosynthetic genes *CPD* and *CYP85A2*. A and B, *LUC* activity measured from the eighth day of development in transgenic seedlings harboring the *CPD:LUC* (A) or *CYP85A2:LUC* (B) transgenes. White and black bars at the time scale indicate light and dark periods, respectively. Zero time is the beginning of the eighth light period. Each section shows the result of a representative measurement. C, Autoradiogram of the RT-PCR products generated from the endogenous *CPD* and constitutive control *UBQ10* transcripts during a daily LD cycle in wild-type seedlings. Zero time is the beginning of the ninth light period.

good agreement with the luminescence levels of the *CPD:LUC* transgenic line (Fig. 1B).

Circadian and Light Regulation of *CPD* Expression

Under LD conditions, the sudden changes of *CPD* expression at lights-on and lights-off suggested a regulatory role of light in control of transcriptional activity. To determine the temporal profile of *CPD* activity in the absence of light signals, we measured the luminescence levels of *CPD:LUC* seedlings in both continuous white light (LL) and continuous dark (DD), following a 7-d LD entrainment period. In LL, the *LUC* activity showed a free-running circadian oscillation

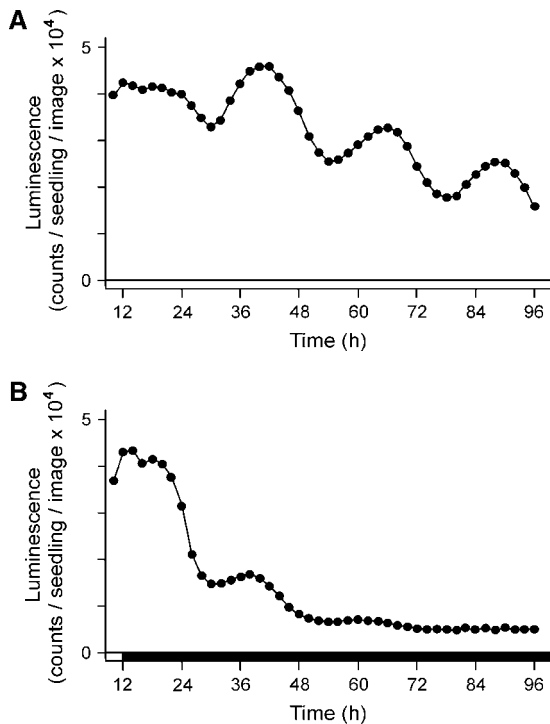


Figure 2. Circadian regulation of *CPD* expression under constant light conditions. A and B, LUC activity measured following 7 d of LD entrainment in transgenic seedlings harboring the *CPD:LUC* transgene during growth in LL (A) or DD (B). Time scale shows zeitgeber time elapsed from the onset of the last (eighth) light period; white and black bars indicate light and dark periods, respectively. Each section shows the result of a representative measurement.

tion that was maintained for several days. The expression cycles had roughly 24-h period lengths and approximately 2-fold rhythmic amplitude, with minimum points at subjective midday and maxima in the subjective night (Fig. 2A). Circadian activity of the *CPD* promoter could also be observed in DD, but under these conditions the cycles became attenuated after 3 d, at which point the expression level decreased to about 10% of the initial value (Fig. 2B).

Compared to the LL activity of *CPD*, the diurnal expression curve shows a strong increase at lights-on and a sudden decrease following lights-off, suggesting a positive role of light in the regulation of *CPD* (Fig. 1A). An activity minimum in the middle of the light phase shows temporal coincidence with the one observed at the subjective light period of the free-running LL cycle (Fig. 2A), and a shoulder in the second half of the dark phase seems to correspond to the postmaximum part of the LL curve measured at the same time of the subjective dark period (Fig. 2A). These similarities between the LD and LL profiles indicate that *CPD* expression is under dual control, with a light induction superimposed upon the circadian oscillation of the transcriptional activity.

Light Induction of *CPD* Is Dependent on Phytochrome Signaling

To clarify which light signaling pathway(s) is involved in the induction of *CPD* expression, we measured the activity of the *CPD:LUC* transgene in LD using light sources of different spectral characteristics. Three samples of seedlings were grown to 1 week old under LD photoperiods, and then their LUC activity was measured in LD cycles with the same light source, or by replacing white illumination with monochromatic red or blue light periods (RD and BD conditions). As shown in Figure 3A, diurnal cycling of *CPD* activity was maintained in RD, and the expression values were very similar to those seen in LD. By contrast, BD regimes caused a decrease in the expression level and severe dampening of the diurnal oscillation, resulting in an activity profile resembling the one detected in DD (Fig. 2B). Because red light was sufficient for enabling diurnal cycles, whereas blue light of the same intensity was not, these data suggest a major role for phytochrome photoreceptors in the light induction of *CPD*.

To verify the importance of phytochrome signaling in the light response, we measured the diurnal expression of *CPD:LUC* in transgenic seedlings lacking functional *PHYA* and *PHYB*, the two most abundant phytochromes. In these seedlings, the luminescence intensity in LD was only about 10% of that measured in wild-type background, and activity changes at lights-on and lights-off were barely recognizable (Fig. 3B). Instead, the LD expression detected in *phyAphyB* plants indicates the dominance of circadian regulation (Fig. 3C). The experiments showing diminished transgene induction by blue light or in phytochrome-deficient background reveal the primary role of phytochrome signaling in the diurnal regulation of *CPD* activity, and also in determining the level of expression.

Diurnal Fluctuation of *CPD* Expression Is Not Determined by Feedback Regulation

The transcription of *CPD* is stringently controlled by the level of biologically active BRs, and in turn the expression of *CPD* can influence BR accumulation. Therefore, it was important to clarify whether the hormonal feedback regulation is involved in the light and/or circadian control of *CPD* expression. To address this question, we measured the expression of the *CPD:LUC* transgene in the BR-insensitive *bri1* mutant that had been shown to express *CPD* at an elevated level (Bancos et al., 2002). We found that, although in LD the activity *CPD:LUC* was about 2-fold higher in *bri1* seedlings than in the wild-type control, the diurnal profile of expression was similar in both transgenic lines (Fig. 4A). Compared to the plants with wild-type background, in the *CPD:LUC*-carrying *bri1* mutant the lights-on response was weaker and slower, whereas the decrease of activity following lights-off was more gradual. These data reveal that diurnal changes in

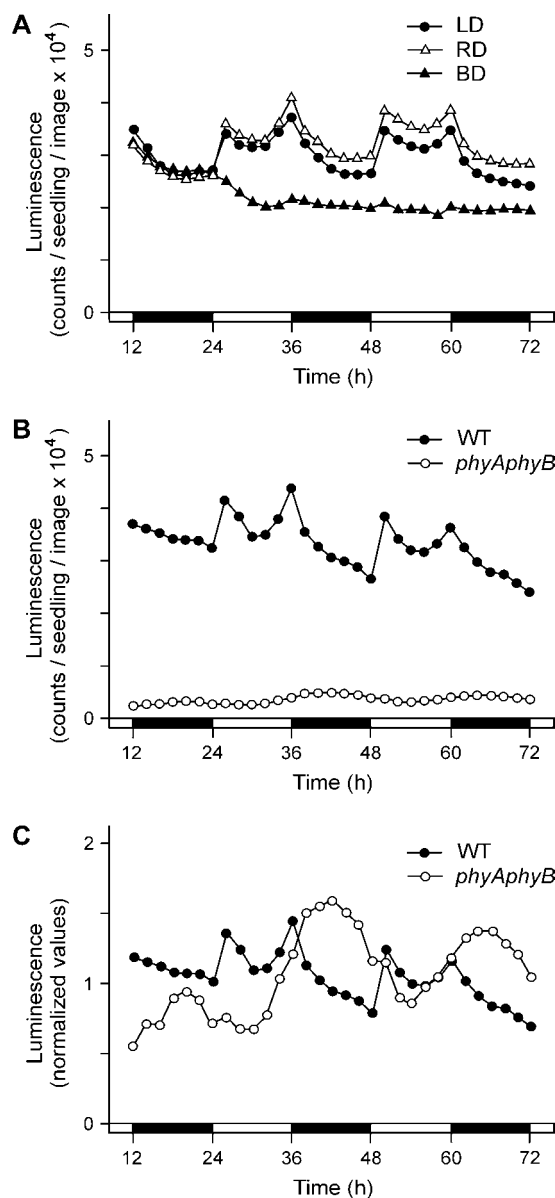


Figure 3. The effects of wavelength and phytochrome deficiency on light induction of *CPD*. A, LUC activity of *CPD:LUC* transgenic plants under photoperiods of different wavelengths. Seedlings were entrained in LD (white light/dark cycles) for 7 d from germination, then grown further under photoperiods of the same length using white, red, or blue light sources (LD, RD, or BD, respectively). Zero time is the beginning of the last (eighth) common white illumination. B, Diurnal *CPD:LUC* transgene activity in wild-type and *phyA-201phyB-5* backgrounds. C, To aid the comparison of the rhythmic component of expression, data from B were normalized for the average luminescence of the individual lines, as detected during the measurements. Zero time is the start of the eighth light period. White and black bars at the time scale indicate white light and dark periods, respectively. Each section shows the result of a representative measurement.

CPD transcript levels do not depend on BR signaling and that the feedback regulation has only a minor modulating effect on the daily expression profile.

We also measured the LL and DD expression of *CPD:LUC* in *bri1* seedlings to find out whether the circadian expression is dependent on BR signaling. In LL a similar oscillation pattern was observed as in the wild-type background, although the amplitude was lower in both absolute and relative terms (Fig. 4B). By

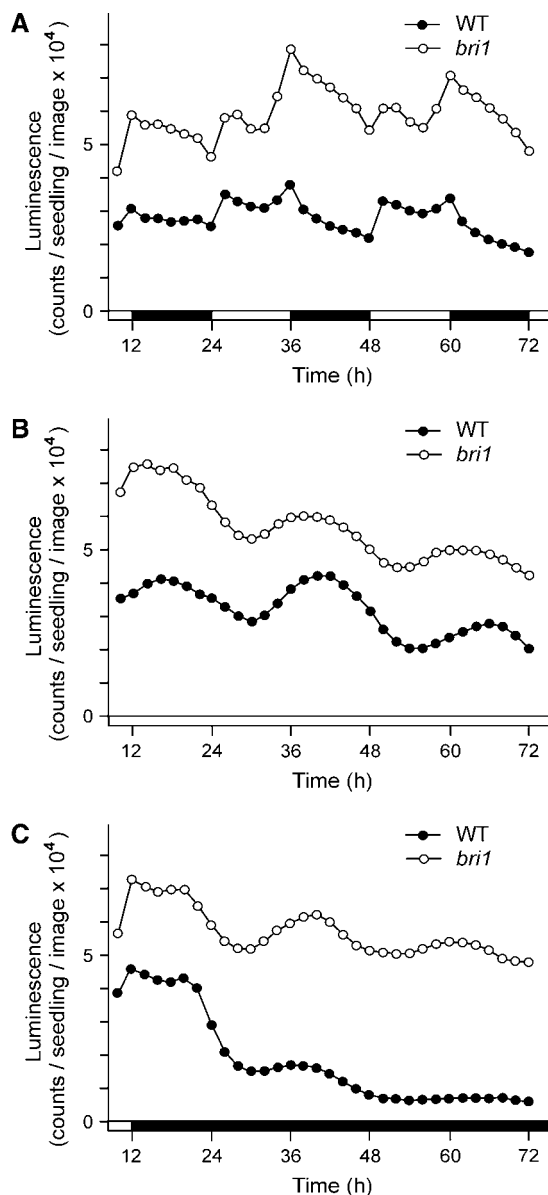


Figure 4. The influence of BR insensitivity on the diurnal and circadian activity of *CPD*. Changes of *CPD:LUC* expression in wild-type or *bri1* backgrounds are shown. Transgenic seedlings were grown in LD for 8 d from germination, and then LUC activity was measured under different light regimes. A, LD; B, LL; C, DD. Zero time is the beginning of the eighth light cycle. White and black bars at the time scale indicate light and dark periods, respectively. Each section shows the result of a representative measurement.

contrast, the activities measured in DD were quite different from those of the wild-type control. Although a gradual dampening of the cycle amplitudes was also observed in *bri1* plants, this was not accompanied by a rapid decrease of the expression level, which remained about as high as under LL conditions (Fig. 4C). These results show that the circadian regulation of *CPD* activity is maintained in the absence of BR signaling, whereas its repression in DD is caused primarily by hormonal feedback regulation.

Diurnal Changes of the Endogenous BR Content

Diurnal oscillation in the expression of BR-biosynthetic genes suggests that the rate of BR synthesis varies during the day/night cycle. To determine whether daily changes of *CPD* and *CYP85A2* expression correlate with the steroid hormone content, we performed quantitative gas chromatography (GC)-mass spectrometry (MS) analysis of the endogenous BRs in 1-week-old wild-type seedlings during the LD cycle at 6-h intervals (Table I). The early C-6 oxidation intermediates cathasterone, teasterone, and 3-dehydro-6-deoxoteasterone were not detectable in the samples taken in the morning just before lights-on, at midday, in the evening before lights-off, and at midnight (0, 6, 12, and 18 h from lights-on, respectively). Levels of the late C-6

oxidation BRs and castasterone showed only minor differences, without any apparent diurnal variation. But, in contrast to the steadiness of the intermediate pools, at midday the seedlings accumulated large amounts of BL, the highly active BR that remained below the detection limit in samples collected at the other three time points of the day. These data show a remarkable increase of the active hormone content at the middle of the light period and also that the synthesis of BL does not appreciably affect the pools of BR intermediates.

Because in DD the expression level of *CPD* decreased rapidly in the wild type but not in the *bri1* background, we also wanted to find out the effect of prolonged dark growth on the endogenous BR content. Wild-type seedlings were grown to 1 week old in LD, then BRs were analyzed in samples that were either placed in darkness for 48 h, starting from the end of the eighth light period, or grown further for the same time in LD. As shown in Table II, the amounts of BR intermediates were only slightly influenced by extended dark treatment, and the level of the bioactive castasterone remained essentially unchanged. These measurements indicate that the repression of *CPD* activity in DD does not the result from an increase of the endogenous BR content.

Feedback Repression of *CPD* Reveals Enhanced BR Responsiveness in the Dark

Our finding that BR-dependent down-regulation of *CPD* expression in DD was not accompanied by an increase of BR levels suggested that light conditions might influence the efficiency of feedback regulation. To find out whether dark repression of *CPD* is controlled by the BZR1 transcription factor, we measured how DD affected the activity of a modified *CPD* promoter (*mCPD*) that contains a point mutation within the BZR1-binding sequence. Seedlings harboring the *mCPD:LUC* fusion accumulated about 10-fold higher amount of the *LUC* mRNA than those with the *CPD:LUC* control, and the transcript level decreased only to 92% of the initial value, opposed to the 8% of the control, upon treatment with 100 nM BL (Fig. 5A). CCD camera measurements revealed that the luminescence profiles of *mCPD:LUC* seedlings in LD and subsequent DD are very similar to those of *CPD:LUC* in the *bri1* background (Fig. 5B). This result shows that depletion of the *CPD* transcript in DD is primarily the consequence of BZR1-mediated feedback regulation. Because in seedlings GC-MS analyses did not detect an increase of the BR content in DD, our data strongly suggest that the feedback response is a result of enhanced BR susceptibility in the dark.

DISCUSSION

De novo synthesis plays an important role in setting the endogenous levels of bioactive BRs. In transgenic

Table I. Endogenous BR levels during a LD cycle

BR data for each time point show the results of three independent experiments, yielding samples of 95 g, 83 g, 80 g, and 98 g (first lane); 37 g, 32 g, 40 g, and 31 g (second lane); and 40 g, 36 g, 40 g, and 37 g (third lane), all fresh weight.

BR	BR Content Hours from Lights-On			
	0	6	12	18
	<i>ng kg⁻¹ fresh weight</i>			
BL	ND ^a	151	ND	ND
	ND	87	ND	ND
	ND	136	ND	ND
Castasterone	47	45	38	46
	72	83	75	64
	73	84	83	64
6-Deoxocastasterone	1,237	1,379	1,470	1,422
	1,114	1,152	1,044	1,242
	1,196	1,156	1,178	1,239
6-Deoxyphasterol	445	491	438	420
	496	495	527	548
	740	412	584	653
3-Dehydro-6-deoxoteasterone	192	232	205	223
	287	275	270	324
	276	318	269	434
6-Deoxoteasterone	68	71	87	66
	182	71	98	77
	80	70	111	79
6-Deoxocathasterone	1,394	1,689	1,638	1,346
	2,124	1,819	1,661	1,756
	2,779	1,856	1,554	1,590

^aND, Not detectable.

Table II. Endogenous BR levels after 48 h in DD

BR data for each time point show the results of three independent experiments, yielding samples of 125 g and 132 g (first lane); 87 g and 109 g (second lane); and 101 g and 100 g (third lane), all fresh weight.

BR	BR Content	
	48 h DD	LD Control
	<i>ng kg⁻¹ fresh weight</i>	
BL	ND ^a	ND
	ND	ND
	ND	ND
Castasterone	79	85
	63	54
	55	65
6-Deoxocastasterone	531	977
	802	905
	891	1,032
6-Deoxyphasterol	387	521
	ND	79
	ND	155
3-Dehydro-6-deoxoteasterone	192	201
	188	182
	220	182
6-Deoxoteasterone	52	49
	143	99
	120	114
6-Deoxocathasterone	1,405	1,608
	887	1,356
	1,336	2,198

^aND, Not detectable.

Arabidopsis, BR biosynthesis could be increased by overexpression of DWF4/CYP90B1, one of the rate-limiting enzymes (Choe et al., 2001), and parallel changes in the amounts of *CPD*: β -glucuronidase and native *CPD* transcripts revealed that the mRNA level of *CPD* is controlled primarily at the level of transcription (Mathur et al., 1998). Accordingly, reporter fusions with promoters of BR-biosynthetic genes, such as those of *CPD*, *CYP85A1*, and *CYP85A2*, could be used successfully to determine developmental and temporal expression patterns, as well as active sites of BR synthesis (Mathur et al., 1998; Castle et al., 2005). Whereas the crucial role and relatively high transcription level of *CPD* make its promoter ideal for in vivo expression assays using LUC reporter, such studies require faithful correspondence between the activities of endogenous *CPD* and the LUC reporter. During the diurnal cycle, we found good agreement between the levels of the *CPD* transcript and *CPD*:LUC-derived luminescence intensity, showing that this reporter construct is a reliable indicator of *CPD* expression.

Our data on the daily changes of *CPD* transcript levels correspond to those detected by a microarray analysis designed to identify diurnally expressed Arabidopsis genes (supplemental data to Schaeffer et al., 2001: genome-www.stanford.edu/microarray). These measurements show transcript accumulation of *CPD* (and also *DET2*, another BR-biosynthetic gene) at the end of the light period. Finer resolution of the diurnal

expression by LUC imaging revealed that dual, light, and circadian regulation of *CPD* leads to two distinct expression maxima during the day: after lights-on and at lights-off. We found that *CYP85A2*:LUC activity follows a similar pattern, suggesting coordinated diurnal control of *CPD* and *CYP85A2* transcription. Although the diurnal amplitude of *CYP85A2* promoter activity was about 2 times higher than that of *CPD*, the

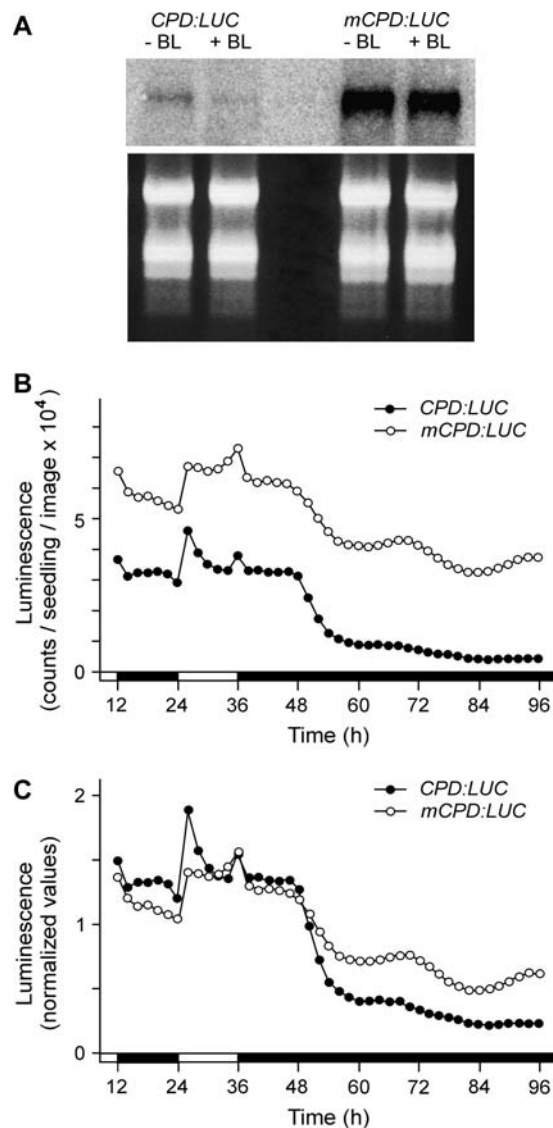


Figure 5. Impaired feedback regulation alleviates dark repression of *CPD*. A, Feedback regulation of the *CPD*:LUC and *mCPD*:LUC transcripts by exogenously applied BL. Northern hybridization and ethidium bromide-stained images of the RNA samples (top and bottom sections, respectively) are shown. B and C, LUC activity of *CPD*:LUC and *mCPD*:LUC transgenic plants. Seedlings were germinated and grown in LD for 8 d, and then LUC activity was measured during LD to DD transition from the end of the eighth light period (zero time). Data from the same experiment are plotted both as absolute (B) and normalized (C) values. White and black bars at the time scale indicate light and dark periods, respectively. B and C show a representative measurement.

5- to 10-fold lower expression level of *CYP85A2* (as indicated by cDNA microarray data available at the Genevestigator Web site: www.genevestigator.ethz.ch; Zimmermann et al., 2004) made its reporter-based detection more erratic.

In *CPD:LUC*-carrying seedlings, LUC activity increased rapidly following lights-on. Whereas the observed light induction is consistent with the concomitant accumulation of *CPD* mRNA, the initial increase can be slightly exaggerated by a weak, nonspecific, and transient enhancement of LUC activity, perhaps resulting from ATP pool expansion, in response to light (Millar et al., 1992). Circadian expression of *CPD*, which peaks in the middle of the subjective night, could be maintained for several days in LL, but decreased and became attenuated in DD in about 48 h. The amplitude of cycling is comparable to that of several circadian-regulated biosynthetic genes in *Arabidopsis* (Harmer et al., 2000), and also of the diurnal changes of *CPD* activity. Both the light induction and circadian control of the *CPD* promoter are functional in the BR-insensitive *bri1* mutant; therefore, these processes are independent of the hormonal feedback regulation. It is tempting to speculate that the diurnal control of transcription may involve the AAAATCT motif present in both the *CPD* and *CYP85A2* promoters. This sequence is a potential binding site of CIRCADIAN CLOCK ASSOCIATED 1, a transcription factor that has been shown to mediate circadian control via both negative and positive transcriptional complexes (Harmer and Kay, 2005), and also phytochrome-regulated gene expression (Green and Tobin, 1999).

Red light-specific induction of *CPD:LUC*, as well as its severely reduced activity and light response in the *phyAphyB* mutant, suggest a primary role for phytochrome signaling in the light regulation of *CPD*. Although in wild-type background we could also observe a slight increase of LUC activity following blue light exposure, which was used within the photobiologically active fluence range (Devlin and Kay, 2000), this may be attributed to PHYA effect or/and the aforementioned nonspecific light-induced increase of LUC activity, rather than cryptochrome-mediated signals. In green seedlings PHYB is the most abundant phytochrome species, whereas PHYA is the least (Sharrock and Clack, 2002). Therefore, the dramatic decrease of light inducibility in plants deficient for both PHYA and PHYB seems to indicate a predominant role for PHYB in controlling *CPD* expression. Light regulation of GAs has been studied in detail. Remarkably, most light-responsive genes of GA synthesis were also found to be under wavelength- or photoreceptor-specific control. In *Arabidopsis*, lettuce (*Lactuca sativa*), and pea (*Pisum sativum*), light was shown to regulate the expression of GA 3 β -hydroxylase and GA 20-oxidase isoforms via either phytochrome-dependent or -independent mechanisms (Toyomasu et al., 1998; Yamaguchi et al., 1998; Ait-Ali et al., 1999; Jackson et al., 2000).

An increasing amount of evidence indicates the involvement of phytohormones in various light-

dependent physiological processes, and in some cases the role of de novo hormone synthesis has been clearly demonstrated. For instance, light was shown to promote the germination of *Arabidopsis* and lettuce seeds by increasing their GA content via the induction of GA 3 β -hydroxylases (Toyomasu et al., 1998; Yamaguchi et al., 1998). Alabadí et al. (2004) reported that both mutational and chemical disruption of GA synthesis can interfere with normal skotomorphogenesis and cause aberrant expression of light-regulated nuclear genes. Furthermore, ethylene and GA were found to act coordinately in the phytochrome-mediated shade avoidance response, but their effect could be abolished by paclobutrazol, an inhibitor of GA synthesis (Pierik et al., 2004).

So far only a few studies have addressed the role of light in influencing the biosynthesis and removal of BRs. In *Arabidopsis*, higher BR levels were detected in light-grown plants than in dark-grown ones, though the plants used in these analyses were not of the same age (Choe et al., 2001). Light-induced accumulation of active BRs was more convincingly demonstrated in light-grown pea seedlings that, compared to the etiolated control, contained 17-fold and 4-fold excess of BL and castasterone, respectively (Symons et al., 2002). Nevertheless, dark-to-light shift of pea seedlings (Symons and Reid, 2003) or light-to-dark shift of *Arabidopsis* seedlings in our experiment did not appreciably change the levels of castasterone and upstream BR intermediates.

Recently, it has been reported that the expression of *Arabidopsis CYP734A1/CYP72B1* and *CYP72C1*, both encoding BR-catabolizing P450s, are light repressed (Turk et al., 2003; Takahashi et al., 2005), indicating the possibility of coordinated regulation of these genes and light-inducible *CPD* and *CYP85A2* that encode biosynthetic enzymes. Light-activated *CYP85A2* and light-repressed *CYP734A1/CYP72B1* and *CYP72C1* expression seem consistent with the observed appearance of BL in young seedlings at midday. However, it remains to be clarified whether diurnal changes of BL synthesis correlate with similar changes in the amount of the *CYP85A2* enzyme. Intriguingly, this transient accumulation of BL does not noticeably affect the pools of BR intermediates, even that of its immediate precursor castasterone, which is synthesized by the same *CYP85A2* enzyme (Kim et al., 2005; Nomura et al., 2005). But castasterone itself serves as substrate for BL synthesis, and in tomato fruit intense BL accumulation was found accompanied by an actual decrease of the castasterone level (Montoya et al., 2005). That light-regulated accumulation of active BRs can contribute to photobiological responses is strongly suggested by reports on reduced phytochrome or light effects in BR-deficient mutants (Luccioni et al., 2002), as well as in transgenic lines that overexpress *CYP734A1/CYP72B1* and *CYP72C1* enzymes (Turk et al., 2005).

The rate of hypocotyl elongation in *Arabidopsis* shows circadian cycling, with maxima at 4 to 6 h after subjective midday (Dowson-Day and Millar, 1999).

Accordingly, an mRNA microarray analysis revealed circadian expression of several genes associated with cell elongation (Harmer et al., 2000). This type of regulation is essential for normal morphogenesis because mutations affecting the circadian clock can also result in abnormal hypocotyl elongation (Dowson-Day and Millar, 1999). The *toc1-3* mutation was shown to elevate the activity of GA5 that encodes a GA 20-oxidase (Blázquez et al., 2002). In potato (*Solanum tuberosum*), the complex diurnal profiles of *StGA20ox1* and *StGA20ox2* expression (Carrera et al., 1999; Jackson et al., 2000) suggest the involvement of circadian regulation. In line with the observed activity changes of GA-biosynthetic genes, the accumulation of bioactive GAs was also found to follow diurnal rhythmicity (Foster and Morgan, 1995). In addition to the daily oscillation of GA levels, the accumulation of elongation-controlling indole-3-acetic acid and ethylene were shown to be determined by the circadian clock (Jouve et al., 1999; Thain et al., 2004). These results imply that diurnal adjustments of phytohormone synthesis are required for normal development, and circadian regulation can ensure proper hormone levels in anticipation of the regular daily changes of environmental conditions. Our data show that the expression of BR-biosynthetic genes and the endogenous BR content are also under circadian control.

Earlier studies revealed that all P450 genes of BR biosynthesis are feedback regulated by bioactive BRs (Bancos et al., 2002; Shimada et al., 2003). Whereas the diurnal expression of *CPD* is only weakly influenced by this control mechanism, we found that the repression of this gene in DD is feedback dependent because it requires BRI1 receptor function and proper binding of the BZR1 repressor to its target site (He et al., 2005) in the *CPD* promoter. That the feedback effect is enhanced in DD without an increase of the BR content strongly suggests that light can decrease BR sensitivity, thereby providing another means of light control over the biosynthesis of BRs. Our finding is in good agreement with earlier reports on different intensities of BR responses under light or dark conditions (Fujioka et al., 1997; Choe et al., 1998; Yang et al., 2005). These regulatory mechanisms of BR synthesis and responsiveness remarkably resemble those of the GAs that also rely on the combination of feedback control of GA 20-oxidases and 3 β -hydroxylases and reduced hormone sensitivity in the light (Reed et al., 1996).

The physiological benefits of diurnally controlled BR synthesis are yet to be elucidated. This complex regulation may ensure optimal hormone levels in accordance with the daily changes in growth and metabolic functions. It may also be important in maintaining BR homeostasis by coordinating oppositely regulated biosynthesis and catabolism during the light and dark periods of the day. Elevated *CPD* and *CYP85A2* expression, as well as BL accumulation, are in apparent contradiction with the reduced hypocotyl elongation during the light periods. This anomaly is likely caused by the observed light-dependent decrease in BR re-

sponsiveness. Our data and earlier reports indicate concerted regulation of BR levels and sensitivity, while flexibility of this dual control may be ensured by developmental and organ-specific differences between these two effects. Although the impact of de novo BR synthesis is not clear, the complex transcriptional regulation of the biosynthetic enzymes indicates its apparent physiological importance.

An interesting possibility is that, by controlling the accumulation of BL during the light phases, diurnal regulation of BR-biosynthetic genes may influence flowering time in Arabidopsis. Recently, it has been shown that intense synthesis of bioactive BRs is associated with reproductive development (Montoya et al., 2005; Nomura et al., 2005; Symons et al., 2006); therefore, it seems feasible that in Arabidopsis light induction of *CYP85A2* can contribute to flower induction through enhancing BL accumulation under long-day conditions. In long-day plants, GAs were reported to promote flowering following long photoperiod up-regulation of light-inducible GA 20-oxidase genes (Blázquez et al., 2002; Lee and Zeevaert, 2002). In Arabidopsis, BRs may have a similar role in determining flower induction because BR-deficient mutants like *det2* show characteristic late flowering phenotype (Chory et al., 1991).

MATERIALS AND METHODS

Plant Material

Seeds of transgenic and wild-type Arabidopsis (*Arabidopsis thaliana*; eco-type Columbia) were surface sterilized and sown on Murashige and Skoog medium (Duchefa) supplemented with 1% (w/v) Suc and 0.2% (w/v) Phytagel (Sigma). Seedlings were grown at 22°C in controlled-environment chambers (SANYO Electronic) under alternating regimes of 12 h white fluorescent light (50–60 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and 12 h dark (LD). LL and DD were provided using the same conditions as in the corresponding phases of LD. Blue and red light emitted by LED panels at wavelengths 470 \pm 5 and 660 \pm 5 nm, respectively, were used at a fluence rate of approximately 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$, approximately equal to their spectral proportions in the white light.

Generation of Transgenic Plants

The *CPD:LUC+* reporter construct was generated by cloning the –968 to –5 segment (relative to the translation start) of the *CPD* promoter (Mathur et al., 1998) into the T-DNA-based binary vector pPCV812 (Koncz et al., 1994), in which the *uidA* (β -glucuronidase) part had been replaced by the firefly (*Photinus pyralis*) LUC-derived *LUC+* (Promega). The *CYP85A2* promoter (–971 to –5) was cloned in the same vector, as was described by Castle et al. (2005). The modified promoter used in the *mCPD:LUC+* was isolated from among PCR-mutagenized derivatives of the *CPD* promoter and, except for a G > A transition at –80 (that strongly inactivates the BZR1-binding sequence CGTGTG by altering its first guanine; He et al., 2005), is identical with the wild-type –968 to –5 segment. The reporter gene-containing binary constructs were conjugated into *Agrobacterium tumefaciens* GV3101 (pMP90RK) as described by Koncz et al. (1994), and stable Arabidopsis transformants were generated in wild-type and *phyA-201phyB-5* (Reed et al., 1994) backgrounds using the flower dip method of Clough and Bent (1998). At least 10 primary transformants were isolated, and from T2 plants showing 3:1 segregation of the transgene homozygous lines were obtained by self-pollination. Following assays of LUC activity, one representative line of each construct was selected for further analysis. (For each reporter construct, the expression profiles of eight primary transformants, including the selected line, are shown in Supplemental Fig. 1.) The *CPD:LUC+* fusion was introduced in *bri1-101*

(Li and Chory, 1997) by crossing this mutant with the selected transgenic line of wild-type background.

Measurement of LUC Activity

Seven-day-old LD-grown transgenic seedlings harboring LUC reporter constructs were transferred to plastic plates with fresh medium. Patches of 100 seedlings were then sprayed twice with filter-sterilized 25 mM D-luciferin (Biosynth A.G.) and 0.01% (v/v) Triton X-100 solution in 5 mM Tris-phosphate buffer, pH 8.0. The last spraying was done 12 h before zero time (the onset of the eighth light period), 24 h before the start of the LUC activity measurements. Alternatively, following surface sterilization, seeds were sown on Murashige and Skoog medium supplemented with 0.5 mM luciferin, placed to 4°C for 12 h, then germinated and raised in DD. Bioluminescence imaging was done at 22°C with a liquid nitrogen-cooled backilluminated digital CCD camera (Princeton Instruments), taking 25-min exposures every second hour. Luminescence intensities were evaluated using Metamorph imaging software (Meta Series 4.5; Universal Imaging). All measurements were repeated at least four times. Since the data were highly reproducible (see Supplemental Fig. 2), in each case we show the result of a representative experiment.

GC-MS Analysis of BR Content

For determining endogenous BR levels during the diurnal cycle, wild-type seedlings were grown in LD, then, starting from the onset of the eighth light period, harvested at 6-h intervals. In three experiments, the plant samples collected at 0 h (subjective morning), 6 h (subjective noon), 12 h (subjective evening), and 18 h (subjective midnight) were immediately frozen in liquid nitrogen, and then subjected to lyophilization. For measuring the BR content in DD, LD-entrained wild-type seedlings were transferred to DD or kept further in LD (control) from the end of the eighth light period (0 h, subjective evening). In three experiments, dark-treated samples and their LD controls were harvested after 48 h (subjective evening), as described above. During and at the end of the dark periods, seedlings were harvested under green safe light.

BR extraction, purification, and analysis were carried out as described by Nomura et al. (2001). In brief, methanol extracts of the plant material were spiked with deuterated BRs prior to reduction to aqueous residues. After partitioning between ethyl acetate and 0.5 M K_2HPO_4 , the former was evaporated to dryness and then partitioned between hexane and 80% methanol. The latter was evaporated and purified using a charcoal column and then a Sephadex LH-20 column. BR fractions were combined and further purified on a Senshu Pak ODS 3251-D column (250- \times 8-mm i.d.). The resulting BR samples were analyzed by GC-MS on a Shimadzu-QP2010 GC-MS system using selected ion monitoring mode.

Transcript Analysis

Total RNA was isolated using TRI reagent (Sigma) as recommended by the manufacturer. For semiquantitative RT-PCR assay, RNA was obtained from the same plant batches that were used for BR analysis during the diurnal cycle. Each cDNA sample was prepared from 5 μ g of RNA using Ready-To-Go T-primed first-strand kit (Pharmacia Biotech). Primers, PCR conditions, and detection of the amplified products derived from the *CPD* and *UBQ10* (constitutive control) transcripts were as described by Bancos et al. (2002). For northern analysis, 20 μ g of RNA samples were separated on formaldehyde-agarose gel (1.2%; w/v) and blotted on Nytran N transfer membrane (Schleicher & Schuell). The RNA blot was hybridized with ^{32}P -labeled probe derived from the full *LUC+* CDS, and following stringent washes the distribution of the label was displayed and quantified using a PhosphorImager 445 SI (Molecular Dynamics).

ACKNOWLEDGMENTS

We thank Katalin Józszai (Institute of Plant Biology, BRC HAS) for her technical support, Yumiko Yamada (Department of Biosciences, Teikyo University) for the purification of plant extracts, Jianming Li (Department of Cellular and Developmental Biology, University of Michigan) for providing the *br1-101* mutant, and Suguru Takatsuto (Department of Chemistry, Joetsu University of Education) for supplying deuterated BR standards.

Received February 13, 2006; revised February 13, 2006; accepted February 28, 2006; published March 10, 2006.

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