A Study of Gibberellin Homeostasis and Cryptochrome-Mediated Blue Light Inhibition of Hypocotyl Elongation[1][W][OA]

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Cryptochromes mediate blue light-dependent photomorphogenic responses, such as inhibition of hypocotyl elongation. To investigate the underlying mechanism, we analyzed a genetic suppressor, *scc7-D* (*suppressors of cry1cry2*), which suppressed the long-hypocotyl phenotype of the *cry1cry2* (*cryptochrome1/cryptochrome2*) mutant in a light-dependent but wavelength-independent manner. *scc7-D* is a gain-of-expression allele of the *GA2ox8* gene encoding a gibberellin (GA)-inactivating enzyme, GA 2-oxidase. Although *scc7-D* is hypersensitive to light, transgenic seedlings expressing GA2ox at a level higher than *scc7-D* showed a constitutive photomorphogenic phenotype, confirming a general role of GA2ox and GA in the suppression of hypocotyl elongation. Prompted by this result, we investigated blue light regulation of mRNA expression of the GA metabolic and catabolic genes. We demonstrated that cryptochromes are required for the blue light regulation of *GA2ox1*, *GA20ox1*, and *GA3ox1* expression in transient induction, continuous illumination, and photoperiodic conditions. The kinetics of cryptochrome induction of *GA2ox1* expression and cryptochrome suppression of *GA20ox1* or *GA3ox1* expression correlate with the cryptochrome-dependent transient reduction of GA_4 in etiolated wild-type seedlings exposed to blue light. Therefore we propose that in deetiolating seedlings, cryptochromes mediate blue light regulation of GA catabolic/metabolic genes, which affect GA levels and hypocotyl elongation. Surprisingly, no significant change in the GA_4 content was detected in the whole shoot samples of the wild-type or *cry1cry2* seedlings grown in the dark or continuous blue light, suggesting that cryptochromes may also regulate GA responsiveness and/or trigger cell- or tissue-specific changes of the level of bioactive GAs.

Cryptochromes are blue light receptors that regulate various photomorphogenic responses in plants, including deetiolation and photoperiodic control of floral initiation (Cashmore, 2003; Lin and Shalitin, 2003). Arabidopsis (*Arabidopsis thaliana*) CRYPTOCHROME1 (CRY1) and CRY2 mediate blue light inhibition of hypocotyl elongation, although CRY1 plays a more prominent role in this response (Koornneef et al., 1980; Ahmad and Cashmore, 1993; Guo et al., 1998; Koornneef et al., 1998; Lin et al., 1998). Cryptochromes and phytochromes regulate many overlapping physiological and developmental responses, and they may do so by regulating similar genes (Fankhauser and Chory, 1997; Quail, 2002; Lin and Shalitin, 2003; Sullivan and Deng, 2003). Many hormones, including auxin, GA, brassinosteroids, ethylene, and cytokinin, are known to be involved in hypocotyl growth (Vandenbussche et al., 2005). In etiolated pea (*Pisum sativum*) seedlings exposed to light, the levels of indole-3-acetic acid, GA, and abscisic acid were found to change to various extents. A reduction of GA_1 (the major bioactive GA in pea) was the first detected and most dramatically changed (Symons and Reid, 2003). Results of genome-wide expression profiling also indicate that many photoreceptor-regulated genes encode enzymes involved in the biosynthesis and catabolism of phytohormones, including GA (Ma et al., 2001; Folta et al., 2003; Ohgishi et al., 2004; Zimmermann et al., 2004; Jiao et al., 2005; X. Yu, D. Shalitin, and C. Lin, unpublished data). Those enzymes are often encoded by multiple-member gene families, but few of those gene families have been characterized in detail sufficient to

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assess the blue light effects on hormonal homeostasis and photomorphogenesis.

GAs are tetracyclic diterpenoid hormones that promote growth, such as hypocotyl elongation (Olszewski et al., 2002; Sun and Gubler, 2004). Only a few of the presently known 126 different GAs have been shown to be physiologically active, including GA_1, GA_3, GA_4, and GA_7 (Hedden and Phillips, 2000). GA_4 is believed to be the major bioactive GA in Arabidopsis. Key enzymes involved in the metabolism and catabolism of bioactive GAs include *ent*-kaurene synthase, P450 monooxygenases, and dioxygenases. Two dioxygenases, GA 20-oxidase (GA20ox) and GA 3β-hydroxygenase (GA3ox), catalyze the last few steps in the synthesis of bioactive GAs (Hedden and Phillips, 2000; Reid et al., 2004). Another dioxygenase, GA 2-oxidase (GA2ox), catalyzes catabolism and inactivation of bioactive GAs or their precursors (Lester et al., 1999; Thomas et al., 1999; Hedden and Phillips, 2000; Schomburg et al., 2003). GA signal transduction involves DELLA proteins; it is believed that GA promotes degradation of DELLA proteins to release their suppression on the GA signaling pathway (Peng and Harberd, 1997; Silverstone et al., 1997; Peng et al., 1999; Olszewski et al., 2002; Sun and Gubler, 2004).

GA is well known for its involvement in phytochrome-regulated hypocotyl elongation (Kamiya and Garcia-Martinez, 1999; Hedden and Phillips, 2000; Garcia-Martinez and Gil, 2001; Halliday and Fankhauser, 2003; Vandenbussche et al., 2005). Phytochromes affect GA levels by regulating expression of the *GA2ox* and *GA3ox* genes (Reid et al., 2002). Phytochromes may also regulate GA responsiveness (Reed et al., 1996; Cao et al., 2005; Foo et al., 2006). It has been recently shown that phyA and phyB mediate light stabilization of the DELLA proteins, which may, at least partially, result from the phytochrome-dependent regulation of GA homeostasis (Achard et al., 2007). In comparison to the phytochrome-regulated responses, the relationship between cryptochromes and GA in the blue light responses is less clear in Arabidopsis. It has been found in pea that cry1 and phyA redundantly regulate *GA2ox* and *GA3ox* expression and GA signaling (Symons and Reid, 2003; Foo et al., 2006). Arabidopsis cry1 has been reported to regulate hypocotyl elongation via its effect on GA and auxin homeostasis or signaling (Folta et al., 2003).

Prompted by a study of the genetic suppressor of *cry1cry2* that corresponds to the Arabidopsis *GA2ox8* gene, we investigated the relationship between GA homeostasis and cryptochrome-mediated deetiolation. We showed that increased expression of a *GA2ox* gene caused hypersensitive or constitutive photomorphogenesis, depending on the relative levels of *GA2ox* overexpression. We further demonstrated that cryptochromes are required for the blue light induction of *GA2ox1* expression and blue light suppression of *GA20ox1* and *GA3ox1* expression. Although all those observations are consistent with a simple hypothesis that cryptochromes may inhibit accumulation of bioactive GAs to suppress hypocotyl growth, our analyses of the GA_4 content in whole shoot samples indicate the involvement of a more complex mode of regulation in the cryptochrome-mediated photomorphogenic responses.

RESULTS

Overexpression of *GA2ox8* Suppresses the Long Hypocotyl Phenotype of the *cry1cry2* Mutant

To investigate the genetic mechanisms underlying cryptochrome-mediated light responses in plants, we prepared an activation-tagging population (see ''Materials and Methods'') in the *cry1cry2* double mutant, which exhibits a long hypocotyl phenotype when grown in blue light (Guo et al., 1998; Mockler et al., 1999). *scc7-D* was one of the dominant mutants identified in this screen. The *scc7-D* mutant suppressed the long-hypocotyl phenotype of the *cry1cry2* parent when grown in blue light, but it showed a normally elongated hypocotyl when grown in the dark (Fig. 1, A–C). However, *scc7-D* is hypersensitive to not only blue light, but also red and far-red (FR) lights (Fig. 1, D–F), suggesting that the corresponding gene is associated with light inhibition of cell elongation mediated by both cryptochromes and phytochromes.

The *scc7-D* locus contains a T-DNA inserted in an intergenic region, at 4,293 bp upstream from the start codon of *GA2ox8* (Fig. 1, G–H), and *GA2ox8* seems the only one overexpressed among the six T-DNA flanking genes tested (Fig. 1I). The light-hypersensitive phenotype of *scc7-D* can be rescued by exogenous GA_3 (Fig. 1J). In addition, *scc7-D* also showed a dwarf and late-flowering phenotype (data not shown). Similar dominant alleles of the *GA2ox8* gene have been reported previously, and it was shown that *GA2ox8* encodes a GA2ox that catalyzes 2β-hydroxylation of C_{20}-GAs (Schomburg et al., 2003). Importantly, plants overexpressing *GA2ox8* contain reduced amounts of bioactive GAs and precursors, while loss-of-function *ga2ox7* and *ga2ox8* mutants exhibited a long hypocotyl phenotype (Schomburg et al., 2003). We concluded that the exaggerated light response in the *scc7-D* allele was also caused by the increased *GA2ox8* expression and reduced accumulation of bioactive GAs.

The level of *GA2ox8* mRNA in *scc7-D* was probably high enough to cause exaggerated hypocotyl inhibition in blue light via phytochromes, some of which can act as blue light receptors, but not high enough to suppress hypocotyl elongation in the absence of light. Indeed, transgenic plants overexpressing the GFP-GA2ox8 fusion protein under the control of a strong 35S promoter exhibited a GA-rescuable constitutive photomorphogenic phenotype (Fig. 2, A–C). Similar to *scc7-D*, the *35S*::GFP-*GA2ox8* seedlings are also hypersensitive to light (Fig. 2, A and B). But contrary to *scc7-D*, the etiolated *35S*::GFP-*GA2ox8* transgenic seedlings grown in the dark also showed short hypocotyls,

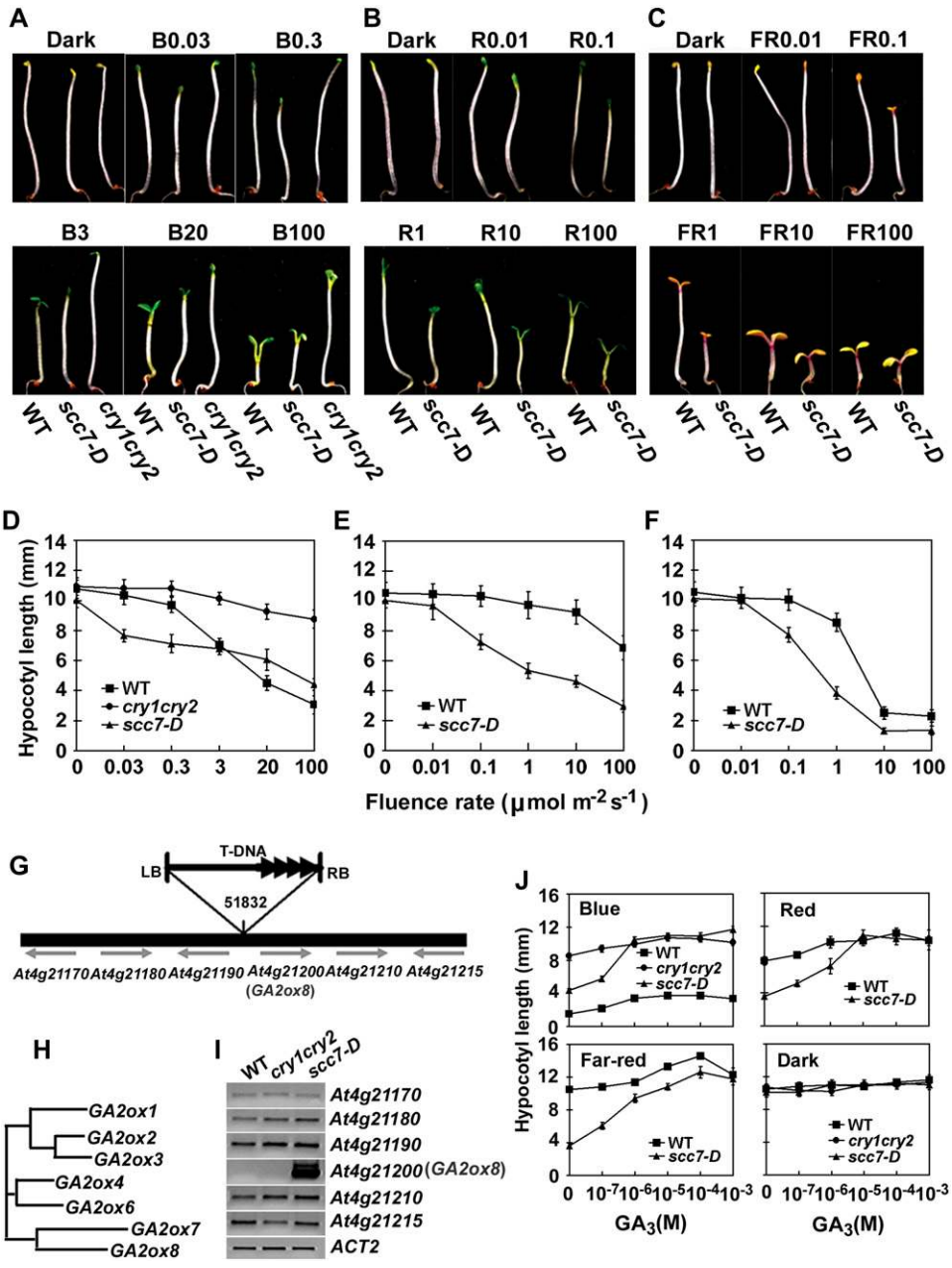

Figure 1. Characterization of the *scc7-D* mutant (A–F). Phenotypic analyses of *scc7-D*. Six-day-old seedlings of *scc7-D*, *cry1cry2* mutant (*cry1cry2*), and wild type (WT) grown in dark or continuous blue (A and D), red (B and E), and FR (C and F) light with indicated fluence rates. Hypocotyl lengths (D–F) and representative seedlings grown under indicated light conditions are shown (A–C). The symbols of B0.03, B0.3, B3, B20, and B100 indicate that seedlings were grown under continuous blue light with fluence rates of 0.03, 0.3, 3, 20, and 100 μmol m^{-2} s^{-1}, respectively. Similarly, R0.01 or FR0.01 symbolize red light or FR light, respectively, with fluence rates (μmol m^{-2} s^{-1}) indicated by the numbers. G to J, Molecular characterization of *scc7-D*. G, Diagram depicting the *scc7-D* locus and the T-DNA insertion. H, A putative phylogenetic relationship of members of the *GA2ox* gene family as predicted using ClustalW (http://www.ebi.ac.uk/clustalw/). I, mRNA expression of genes flanking the T-DNA insert of *scc7-D*. J, Hypocotyl lengths of *scc7-D* seedlings grown in the dark or in continuous lights of different wavelength (100 μmol m^{-2} s^{-1} blue or red light, and 1 μmol m^{-2} s^{-1} FR light) in the presence of different concentrations of GA_3.

as well as unhooked and partially opened cotyledons (Fig. 2, A and B). The phenotype of *35S*::GFP-*GA2ox8* was rescued by exogenous GA_3 (Fig. 2C; data not shown). The levels of the *35S*::*GFP-GA2ox8* mRNA were markedly higher in transgenic *35S*::*GFP-GA2ox8* seedlings than that of the *GA2ox8* mRNA in *scc7-D* or wild-type seedlings (Fig. 2D), which explains why *35S*::*GFP-GA2ox8* lines are constitutively photomorphogenic regardless of light whereas *scc7-D* seedlings showed exaggerated photomorphogenic response only in light. Our observation that overexpression of a *GA2ox* enzyme, which catalyzes inactivation of GAs, caused constitutive photomorphogenesis is consistent with a previous report that the *ga1* mutant impaired in the *ent-copalyl diphosphate synthase* gene showed constitutive photomorphogenesis (Sun et al., 1992; Alabadi et al., 2004). Both observations suggest that photomorphogenic development is at least partially regulated by the levels of bioactive GA.

Association between GA Homeostasis and Blue Light Inhibition of Hypocotyl Elongation

The study of *scc7-D* prompted us to further explore a possible association between cryptochrome function and GA homeostasis. The wild-type and the *cry1cry2* mutants (grown at 22°C) responded only weakly to bioactive GA_3 or GA_4, regardless of light conditions

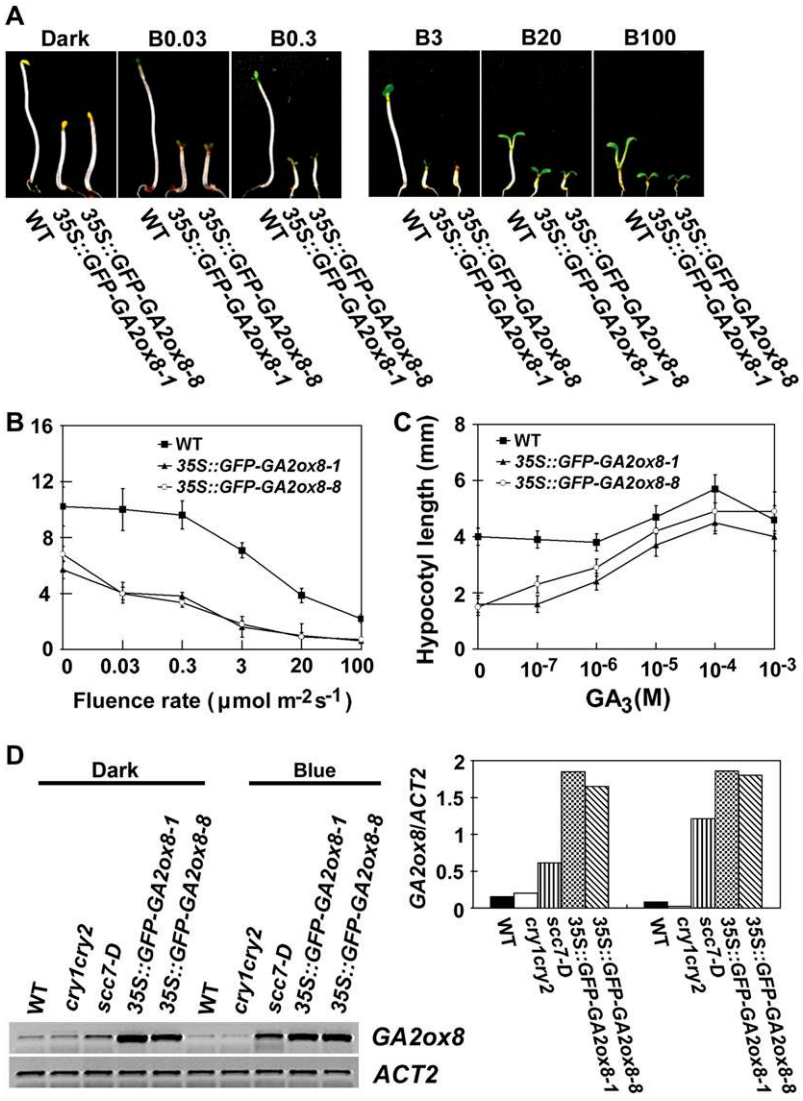

Figure 2. The 35S::GFP-GA2ox8 transgenic plants are constitutively photomorphogenic (A–C). GA-rescuable constitutive photomorphogenic phenotype of two independent transgenic lines overexpressing the GFP-GA2ox8 protein. D, A markedly higher level of *GFP-GA2ox* mRNA in the *35S::GFP-GA2ox8* transgenic plants than the level of *GA2ox8* mRNA in *scc7-D*. Seedlings were grown in the dark or continuous blue light for 6 d.

(Fig. 3). Even under the highest fluence rate of blue light tested, whereby the wild-type seedlings normally exhibited minimum hypocotyl elongation, high concentrations of GA_3 or GA_4 (0.1–1 mM) failed to elicit a large increase in hypocotyl growth or to phenocopy the *cry1cry2* mutant in the wild-type seedlings (Fig. 3, A and E). It is noteworthy that although various hormones have been shown to antagonize light inhibition of hypocotyl growth when applied exogenously, such treatments never completely reverse the inhibitory effect of light on growth (Vandenbussche et al., 2005). At a higher temperature (26°C), the wild-type Arabidopsis seedlings responded relatively more strongly to exogenous GAs as reported previously (Fig. 3F; Collett et al., 2000). A thermoperiodic regulation of *PsGA2ox* expression has been reported to control thermoperiodic fluctuation of GA_1 content and stem elongation of pea stems (Stavang et al., 2005). Whether our observation represents a temperature-sensitive GA responsiveness or that it may be related to the high temperature promotion of auxin-mediated hypocotyl elongation (Gray et al., 1998) remains to be further investigated.

We next examined how the *cry1cry2* mutant responded to GA biosynthesis inhibitors. The GA biosynthesis inhibitors, paclobutrazol (Fig. 3B) or ancymidol (Fig. 3D), can both rescue the long-hypocotyl phenotype of the *cry1cry2* mutant, which is consistent with the observation of the *cry1* mutant (Folta et al., 2003). These results suggest that GA is likely involved in the development of the long hypocotyl of *cry1cry2*, and that the level of bioactive GA is important for cryptochrome function. It is interesting that, when GA synthesis was inhibited, the *cry1cry2* mutant seedlings grown in blue light with relatively high fluence rates exhibited greater hypocotyl elongation in response to GA_3 than the wild-type seedlings (Fig. 3C; B3, B20, B100). These results indicate that cryptochromes may

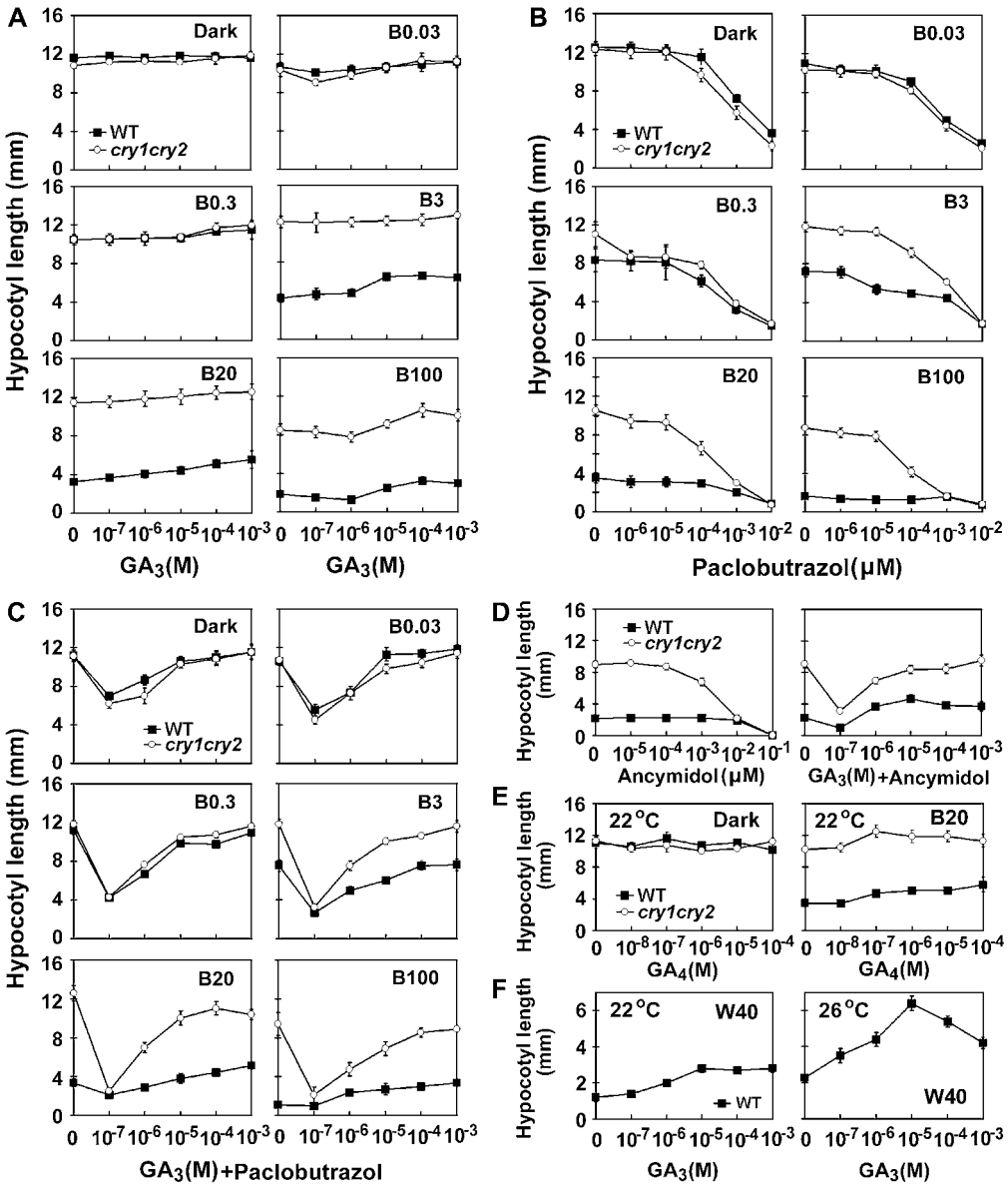

Figure 3. Effects of GAs or GA biosynthesis inhibitors on blue light inhibition of hypocotyl elongation. Seedlings were grown on Murashige and Skoog medium containing different concentrations of GA_3 (A), GA_4 (E), GA inhibitors paclobutrazol (B) or ancymidol (D), and GA inhibitors plus GA_3 at different concentrations (C and D). The GA inhibitor used in C or D is paclobutrazol (0.02 μM) or ancymidol (0.1 μM), respectively. Hypocotyl lengths of 6-d-old seedlings grown under blue light of different fluence rates are shown. The symbols of B0.03, B0.3, B3, B20, and B100 indicate that seedlings were grown under continuous blue light with fluence rates of 0.03, 0.3, 3, 20, and 100 μmol m^{-2} s^{-1}, respectively. Hypocotyl responses of seedlings grown on Murashige and Skoog medium containing different concentrations of GA_3 under white light (W40, 40 μmol m^{-2} s^{-1}) at two different temperatures are shown in F.

also be required to suppress GA response under blue light.

Cryptochromes Mediate Blue Light-Activated mRNA Expression of *GA2ox* Genes, Especially *GA2ox1*, under Dark-to-Light Transfer, Continuous Illumination, and Photoperiodic Conditions

The Arabidopsis genome encodes up to eight *GA2ox*-related sequences, referred to as *GA2ox1* to *GA2ox8* (see "Materials and Methods"). However, the mRNA expression of only six members (*GA2ox1*, *GA2ox2*, *GA2ox4*, *GA2ox6*, *GA2ox7*, and *GA2ox8*) was readily detected in our study. *GA2ox5* seems to be a pseudogene (Hedden et al., 2001; Schomburg et al., 2003), whereas *GA2ox3* is normally expressed at a low level in the absence of auxin induction (Frigerio et al., 2006). Results of various DNA microarray studies concerning the blue light effect on *GA2ox* expression were not always consistent. For example, the expression of

GA2ox1 shows an 8-fold increase in response to blue light in the wild-type seedlings in one study (https://www.genevestigator.ethz.ch), but a 2.4-fold increase of *GA2ox1* transcript was reported in the *cry1* mutant in another study (Folta et al., 2003). We decided to systematically examine the blue light and cryptochrome effects on the expression of each member of the *GA2ox* gene family upon transfer from dark to blue light and under photoperiodic and continuous light conditions.

We first analyzed blue light regulation of *GA2ox* expression in response to inductive blue light treatment. In this experiment, 6-d-old etiolated seedlings were exposed to blue light (100 μmol m^{-2} s^{-1}) for 0.5 to 24 h, and the level of mRNA expression of the *GA2ox* genes was examined at various time points. In wild-type seedlings, all six *GA2ox* genes tested showed various degrees of blue light induction of mRNA expression (Fig. 4). For example, the expression of *GA2ox1* and *GA2ox8* increased about 2- to 3-fold within 60 min of blue light treatment, or about 6- to 8-fold within 8 h of blue light treatment, respectively (Fig. 4B). The blue light-induced mRNA expression is transient for most *GA2ox* genes tested. The expression generally increased immediately after blue light treatment, peaked within 12 h, and returned to the dark level within 24 h of blue light treatment. Four of the *GA2ox* genes tested (*GA2ox1*, *GA2ox2*, *GA2ox6*, and *GA2ox8*) showed reduced blue light induction in the *cry1* or *cry1cry2* mutant (Fig. 4; data not shown). The blue light induction of *GA2ox1* expression was partially impaired in the *cry1* mutant (data not shown), but it was almost completely abolished in the *cry1cry2* mutant (Fig. 4). Our results are consistent with a previous DNA microarray study by Thomas Kretsch and colleagues (http://www.uni-tuebingen.de/plantphys/

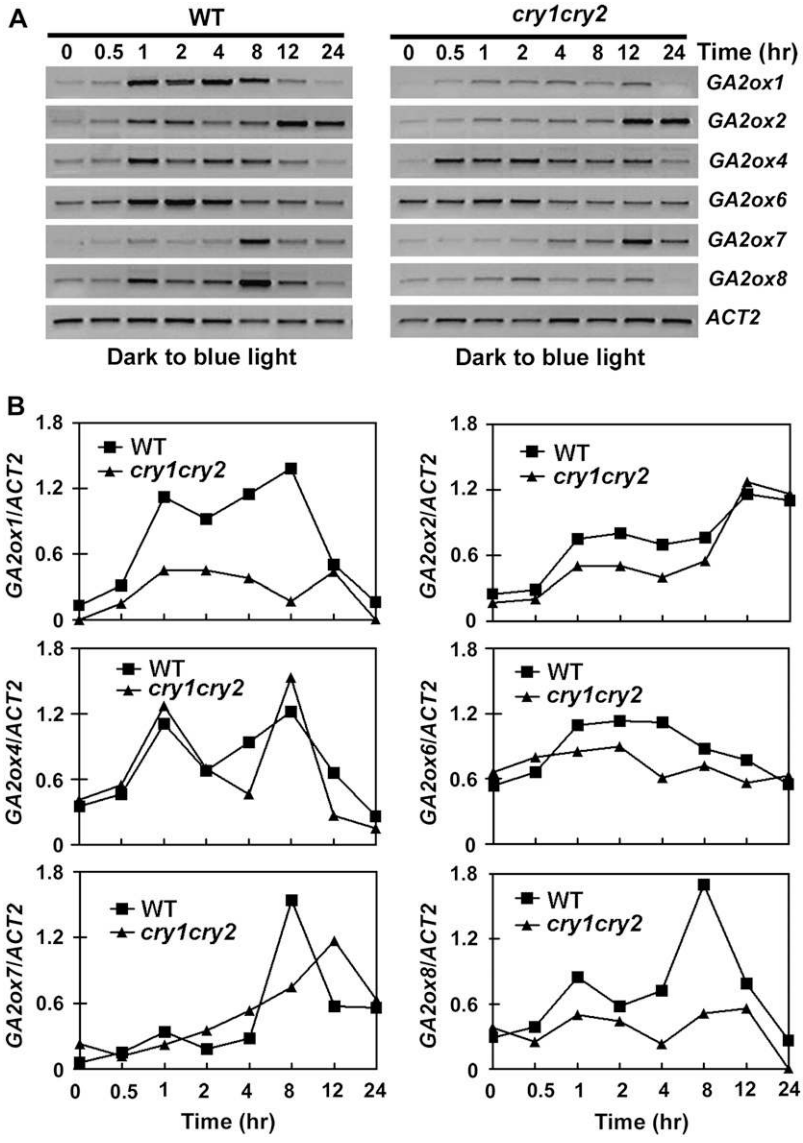

Figure 4. Blue light-induced change of mRNA expression of the *GA2ox* genes. Six-day-old etiolated wild-type or *cry1cry2* mutant seedlings were exposed to 100 μmol m^{-2} s^{-1} blue light, and samples were collected at the time points indicated for RNA analyses. Levels of mRNA expression are shown as the RT-PCR gel images (A) and the relative signal intensities (B).

AFGN/atgenex.htm) but opposite to the result of another DNA microarray study (Folta et al., 2003). It is particularly interesting that *GA2ox1* is much more responsive to cryptochrome-dependent blue light induction than other *GA2ox* genes, because *GA2ox1* is one of the *GA2ox* genes that are not induced by auxin (Frigerio et al., 2006). The observation that *GA2ox1* is more responsive to blue light than other auxin-inducible *GA2ox* genes indicates that *GA2ox1* may be associated with the auxin-independent growth response reported previously (Collett et al., 2000).

The transient nature of the blue light induction of *GA2ox* expression observed in the 24 h inductive blue light treatment (Fig. 4) may be due to the diurnal or circadian rhythmic expression of the *GA2ox* genes. To test this possibility, seedlings were entrained in long-day (16-h light/8-h dark) and short-day (8-h light/16-h dark) photoperiods for 10 d and then transferred to continuous white light for 2 d. Samples were collected every 3 h for 1 d before transfer and 2 d after transfer to the free-running condition, and the expression of each *GA2ox* gene was analyzed. The mRNA expression of all six *GA2ox* genes exhibited either a diurnal rhythm or circadian rhythm (Fig. 5; data not shown). Among them, *GA2ox1* and *GA2ox2* showed the most robust circadian rhythms that were sustained in seedlings transferred from the long-day or short-day photoperiod to continuous white light for at least 2 d (Fig. 5, A and B; data not shown). The phase of the circadian rhythm of *GA2ox2* was dramatically different in plants grown in long-day and short-day photoperiods (Fig. 5B). It has been shown recently that GA_4 is the active GA regulating floral initiation (Eriksson et al., 2006). Whether the photoperiod-dependent phase change of *GA2ox2* expression affects photoperiodic flowering in Arabidopsis remains to be further investigated (Blazquez and Weigel, 1999; Yu et al., 2006; Zhao et al., 2007). The high-amplitude rhythmic expression of *GA2ox1* was all but abolished in the *cry1cry2* mutant grown in long-day photoperiod (Fig. 5, C and D). The similar pattern of changes of *GA2ox1* expression in the *cry1-cry2* mutant shown in Figures 4B and 5D indicate that the transient nature of the blue light induction of *GA2ox1* expression is at least partially attributed to its rhythmic expression. It is also noteworthy that although *GA2ox1* expression is regulated by both cryptochrome and phytochrome, cryptochrome alone had significant effect on the *GA2ox1* expression in seedlings grown in white light under the photoperiodic condition (Fig. 5D).

We next compared the steady-state levels of *GA2ox* expression in seedlings grown in continuous blue light or dark (Fig. 6). Four *GA2ox* genes (*GA2ox1*, *GA2ox2*, *GA2ox4*, *GA2ox7*) tested showed higher steady-state levels of mRNA in seedlings grown in continuous blue light than in etiolated seedlings (Fig. 6A). Under continuous blue light, the expression of *GA2ox1* showed a more pronounced blue light response than other *GA2ox* genes; the mRNA expression of *GA2ox1* was at least 7-fold higher in light-grown wild-type seedlings than that in etiolated wild-type seedlings (Fig. 6A). A more pronounced blue light effect on the

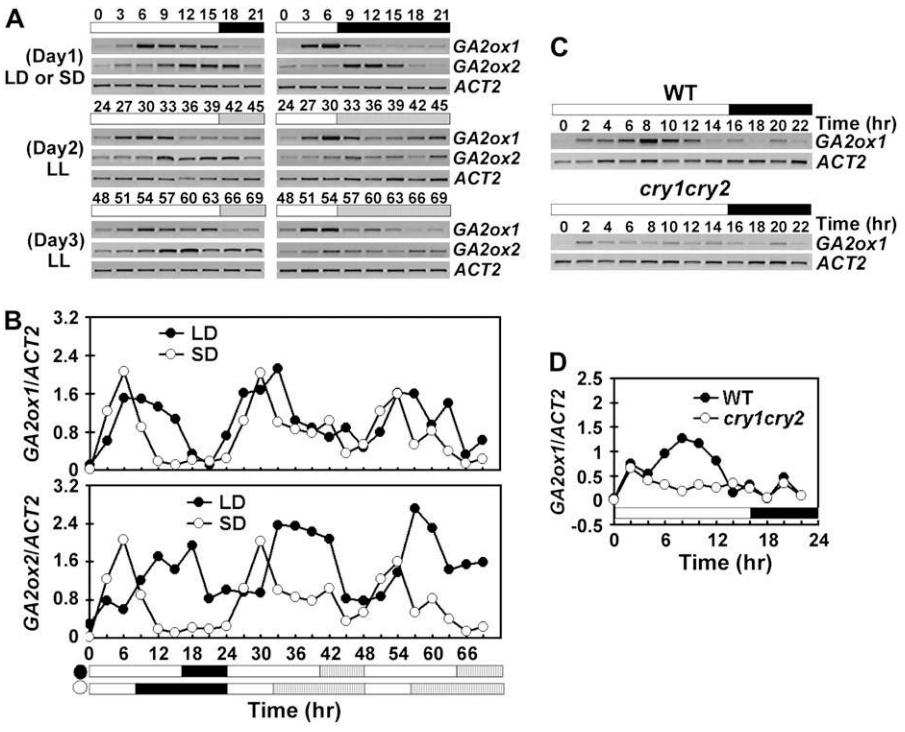

Figure 5. Cryptochromes control the circadian rhythm of *GA2ox1* mRNA expression. mRNA expression of *GA2ox1* and *GA2ox2* genes in seedlings grown under long day (LD) or short day (SD) for 10 d and then transferred to continuous white light were examined using RT-PCR. Samples were collected every 3 h for 1 d in photoperiod and 2 d in continuous white light (A and B), or every 2 h for 1 d in long-day photoperiod (C and D). The white/black bars indicate light/dark phases, and the dashed bars indicate subjective night phase under continuous light. The time (hour) of light on of the first day of sample collection is set as zero.

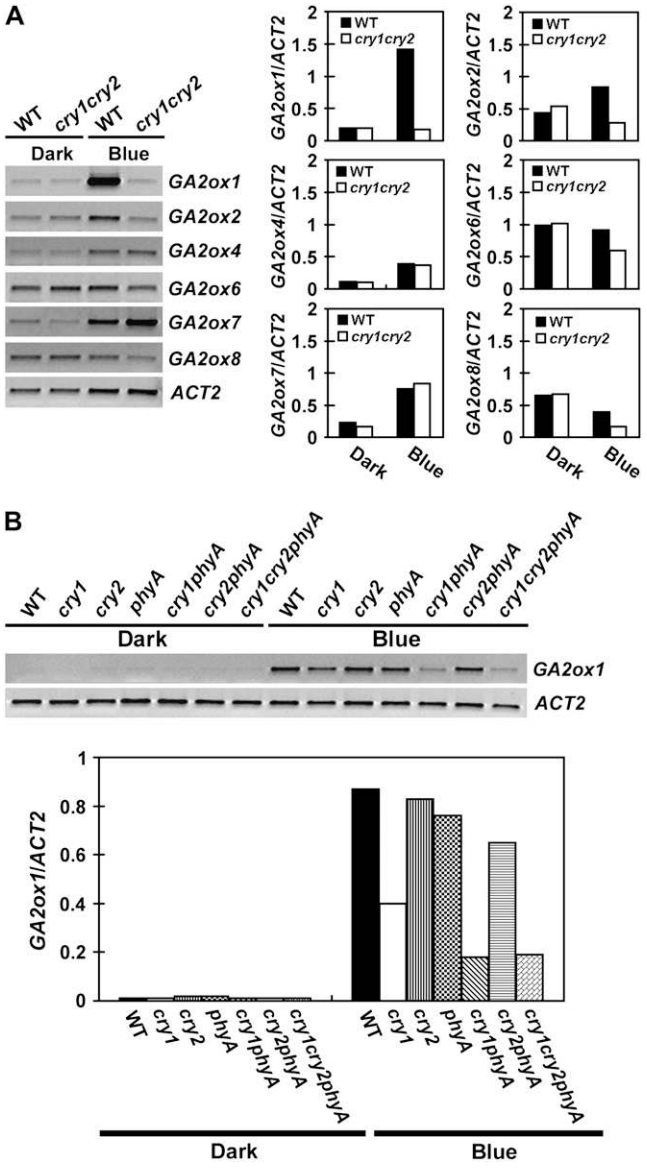

Figure 6. Comparison of the level of *GA2ox* mRNAs in seedlings grown in dark or continuous blue light. Six-day-old seedlings were grown in dark or continuous blue light (20 μmol m^{-2} s^{-1}), the levels of mRNA expression are shown as the RT-PCR gel images (left) and the relative signal intensities (right). The mRNA expression of *GA2ox* genes in the wild-type or *cry1cry2* mutant are shown in A. An independent experiment shows the levels of *GA2ox1* mRNA expression in 6-day-old wild type and the indicated photoreceptor mutants grown in the dark or continuous blue light (B).

GA2ox1 expression is consistent with that observed under other conditions (Figs. 4 and 5) and that found in continuous white light (Achard et al., 2007). Although our study was initially prompted by the observation that overexpression of *GA2ox8* suppressed *cry1cry2* phenotype in the *scc7-D* mutant (Fig. 1), the level of *GA2ox8* mRNA expression was not dramatically affected by continuous blue light or cryptochromes (Figs. 2D and 6B). The blue light-induced *GA2ox1* expression in seedlings grown under continuous blue light was moderately impaired in the *cry1* mutant (Fig. 6B), but it was markedly reduced in the *cry1cry2* (Fig. 6A), *cry1phyA* (Fig. 6B), and *cry1cry2-phyA* mutants (Fig. 6B). These results demonstrate that CRY1 acts redundantly with CRY2 or phyA to mediate blue light induction of *GA2ox1* expression. We concluded that cryptochromes are the major blue light receptors required for the transient blue light induction of *GA2ox1* expression in etiolated seedlings exposed to blue light, for the circadian rhythmic *GA2ox1* expression in seedlings grown in long-day photoperiods under white light, and for the high level steady-state mRNA expression of *GA2ox1* in seedlings grown in continuous blue light.

Cryptochromes Mediate Blue Light Suppression of *GA20ox1* and *GA3ox1* mRNA Expression

In addition to the GA2ox enzymes that catalyze catabolism or inactivation of bioactive GAs such as GA_4, two other dioxygenases, GA20ox and GA3ox, that catalyze synthesis of bioactive GAs are also critical to GA homeostasis (Hedden and Phillips, 2000; Reid et al., 2004). Therefore, we next examined blue light and cryptochrome effects on the expression of *GA20ox* and *GA3ox* genes. Figure 7 shows that expression of the three *GA20ox* genes and one of the two *GA3ox* genes tested were lower (to differing extents) in seedlings grown in continuous blue light than in etiolated seedlings (Fig. 7A). The blue light suppression of *GA20ox1* and *GA3ox1* expression are dependent on cryptochromes, because the sustained blue light suppression of both genes was largely abolished in the *cry1cry2* mutant (Fig. 7). Therefore, *GA20ox1* and *GA3ox1* are also major targets of cryptochromes. Similar to what was observed for the *GA2ox* genes (Fig. 4), but in the opposite direction, the expression of most *GA20ox* and *GA3ox* genes tested (except *GA3ox2*) showed a transient decrease in etiolated seedlings exposed to blue light (Supplemental Fig. S1). The blue light suppression of *GA20ox1* and *GA3ox1* expression was moderately affected in the *cry1cry2* mutant upon transfer from dark to blue light, although their expression was more significantly affected in continuous blue light (Fig. 7; Supplemental Fig. S1). In contrast to all the other GA biosynthesis genes tested, *GA3ox2* expression exhibited cryptochrome-dependent blue light induction upon transfer from dark to blue light and under continuous light conditions (Fig. 7; Supplemental Fig. S1). In contrast to all the other GA biosynthesis genes tested, *GA3ox2* expression exhibited blue light induction upon transfer from dark to blue light and under continuous light conditions, and it was impaired in the *cry1cry2* mutant only under the inductive condition (Fig. 7; Supplemental Fig. S1). It is known that phyB mediates red light activation of *GA3ox2* expression in germinating seeds to facilitate germination (Yamaguchi et al., 1998). The blue light

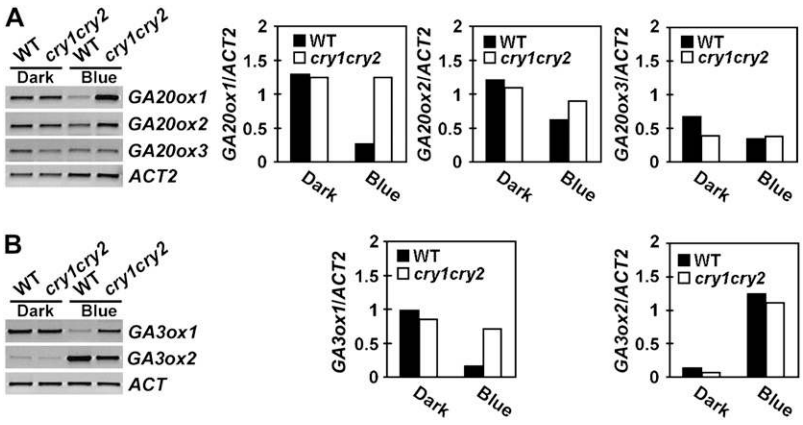

Figure 7. Expression of *GA20ox* and GA3ox genes in seedlings grown in the dark or continuous blue light. Six-day-old wild-type or *cry1cry2* mutant seedlings grown in dark or continuous blue light (20 μmol m^{-2} s^{-1}) were analyzed for the expression of the *GA20ox* genes (A) or the *GA3ox* genes (B). Levels of mRNA expression are shown as the RT-PCR gel images (left) or the relative signal intensities (right).

activation of *GA3ox2* expression may also be associated with light stimulation of cotyledon expansion rather than light inhibition of hypocotyl elongation, but this response appears redundantly regulated by phytochromes and cryptochromes. We might predict that overall, the cryptochrome-dependent blue light suppression of the expression of GA biosynthesis genes such as *GA20ox1* and *GA3ox1*, and cryptochrome-dependent blue light activation of the expression of GA catabolic genes such as *GA2ox1*, may cause a reduction in bioactive GA levels. However, the discrepancy regarding *GA3ox2* expression (discussed above) and the relatively small effect of blue light on the expression of the *GA20ox2*, *GA20ox3*, *GA2ox2*, *GA2ox6*, and *GA2ox8* genes make such predictions very uncertain. Direct measurement of endogenous bioactive GAs is clearly required to accurately assess the effect of blue light on GA level.

Cryptochromes Mediate Transient Blue Light Suppression of GA_4 Accumulation

To test whether cryptochrome-mediated blue light regulation of gene expression changes correlate with changes in the level of bioactive GA_4, we examined GA_4 levels in etiolated wild-type and *cry1cry2* mutant seedlings exposed to blue light. As expected, the level of GA_4 showed a statistically significant decline in the wild-type seedlings exposed to blue light for 4 h (Fig. 8A). The blue light-induced decrease of GA_4 was dependent on cryptochromes, because no statistically significant reduction of GA_4 was detected in the *cry1cry2* mutant (Fig. 8A). After a blue light treatment for 24 h, the level of GA_4 in the etiolated wild-type seedlings exposed to blue light became indistinguishable from that in the etiolated seedlings (Fig. 8A), suggesting the cryptochrome-dependent blue light inhibition of GA_4 accumulation was transient or rhythmic. The transient decrease of GA_4 upon transfer from dark to blue light appears to correlate with the transient (or rhythmic) increase of *GA2ox1* expression (Figs. 4 and 5) and transient (or rhythmic) decrease of *GA20ox1* and *GA3ox1* expression (Supplemental Figs. S1 and S2). A transient decline of bioactive GA accumulation in etiolated seedlings exposed to blue light has also been reported previously in pea (Foo et al., 2006). In this case the major bioactive GA in pea, GA_1, showed a significant but transient decrease in etiolated seedlings exposed to blue light. In contrast to Arabidopsis, wherein cryptochromes mediate the transient blue light suppression of GA_4, cry1 and phyA redundantly control the transient blue light suppression of GA_1 in pea (Foo et al., 2006).

Finally, we compared GA_4 levels in etiolated wild-type and *cry1cry2* seedlings with those grown under continuous blue light. Interestingly, we detected no significant difference in GA_4 level in those samples (Fig. 8B). However, because whole-shoot samples were used in our GA analyses, we cannot exclude the possibility that a localized or cell-specific change of GA_4 content may occur in seedlings grown in continuous blue light.

DISCUSSION

In this study, we showed that increased expression of a *GA2ox* gene genetically suppressed the *cry1cry2* mutant (Figs. 1 and 2). Although this observation by itself may have alternative interpretations, our follow-up photophysiological (Fig. 3) and gene expression studies (Figs. 4–5) demonstrate that cryptochromes are indeed positive regulators of *GA2ox* genes, especially *GA2ox1*. We showed that cryptochromes are required for the transient induction of *GA2ox1* expression in etiolated seedlings exposed to blue light, for the sustained elevation of *GA2ox1* expression in seedlings grown in continuous blue light, and for maintaining a high amplitude of the circadian rhythm of *GA2ox1* expression in seedlings grown in long-day photoperiods. Consistent with the cryptochrome-mediated blue light stimulation of the expression of the GA catabolic gene *GA2ox*1, we also demonstrated that cryptochromes mediate blue light suppression of the

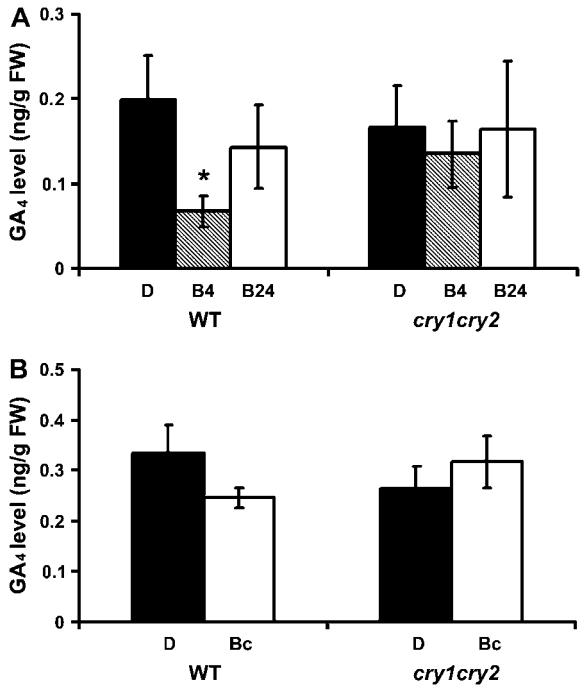

Figure 8. Cryptochromes mediate transient blue light suppression of GA_4. The GA_4 content of 6-d-old etiolated wild-type (WT) and *cry1cry2* mutant seedlings exposed to blue light (60 μmol m^{-2} s^{-1}) for 4 or 24 h are shown in A. The GA_4 content of 5-d-old wild-type (WT) and *cry1cry2* mutant seedlings grown in continuous dark or continuous blue light are shown in B. Asterisk indicates a significant difference ($P <$ 0.05, two-way ANOVA). D, Dark; B4 and B24, etiolated seedlings exposed to blue light for 4 and 24 h, respectively; Bc, seedlings grown in continuous blue light. GA_4 in whole seedling samples except roots was separated by HPLC and analyzed by gas chromatography-MS.

expression of GA biosynthesis genes *GA20ox1* and *GA3ox1* (Fig. 7). We concluded that cryptochromes are positive regulators of *GA2ox1* but negative regulators of *GA20ox1* and *GA3ox1*. These cryptochrome-regulated gene expression changes may result in a blue light-dependent reduction of bioactive GAs. Given the well-established role of GA as a growth promoter, we propose that cryptochrome-regulated change in GA homeostasis is an important mechanism underlying blue light inhibition of hypocotyl elongation.

Consistent with this hypothesis, our analyses of GA_4 content showed a cryptochrome-dependent transient reduction of GA_4 in etiolated wild-type seedlings exposed to blue light (Fig. 8A). This result correlates with the transitory/rhythmic expression patterns of many GA metabolism/catabolism genes (Figs. 4 and 5; Supplemental Figs. S1 and S2). However, in contrast to the transient reduction in GA levels in deetiolating seedlings exposed to blue light, we did not detect a significant reduction of GA_4 in the wild-type seedlings grown in continuous blue light (Fig. 8B) or white light (G.M. Symons and J.B. Reid, unpublished data). Neither did we detect a significant effect of the *cry1cry2* mutation on the GA_4 level in seedlings grown in continuous blue light (Fig. 8B). These results impose a significant challenge to the hypothesis that cryptochromes inhibits hypocotyl elongation solely by reducing GA_4 levels. Similar observations have been previously reported in pea (O'Neill et al., 2000; Symons and Reid, 2003; Foo et al., 2006). In comparison to etiolated seedlings, the level of bioactive GA also showed a transient reduction in pea seedlings transferred from dark to blue light but not in seedlings grown under prolonged illumination (Reid et al., 2002; Foo et al., 2006). It was hypothesized that phyA and cry1 regulate not only GA homeostasis but also GA responsiveness to affect shoot elongation (Reid et al., 2002; Foo et al., 2006). Therefore, it is possible that light regulation of GA responsiveness or GA signal transduction may account for the inhibition of hypocotyl elongation in Arabidopsis plants grown under continuous blue light.

However, we cannot escape the question why continuous blue light caused markedly changed mRNA expression of the *GA2ox1*, *GA20ox1*, and *GA3ox1* genes (Figs. 4–7) without a significant change in the level of GA_4 (Fig. 8B). It would be interesting to examine whether those mRNA changes actually resulted in corresponding changes in the protein levels of the respective key enzymes in GA homeostasis. Alternatively, a localized change of GA_4 levels in response to continuous blue light may provide another possible explanation of our puzzling observations. Specifically, a blue light-dependent reduction of GA_4 may be limited to specific organs or cells. For example, light inhibits hypocotyl elongation but stimulates cotyledon expansion, suggesting that light may trigger a decrease or increase of GA_4 in hypocotyls or cotyledons, respectively. Moreover, hypocotyl elongation is accomplished mostly by a limited number of cells in the elongation zone (Vandenbussche et al., 2005). Therefore, it is conceivable that light may suppress GA_4 accumulation only in cells located in the elongation zone, but not in cells located in other regions of the same hypocotyl. Such localized changes of GA_4 homeostasis may not be readily discernable when the whole shoot samples were examined as in this study. The localized change of hormone homeostasis is not uncommon in plants. For example, differential distribution of GA_1 was previously reported in pea seedlings (O'Neill et al., 2000). Developmental regulation by cell-specific auxin and cytokinin biosynthesis have also been reported recently in Arabidopsis and rice (*Oryza sativa*), respectively (Cheng et al., 2006; Kurakawa et al., 2007). A cell-specific change of GA levels in response to blue light may be regulated by a post-translational mechanism specific to the respective cells, because the blue light-dependent changes of mRNA expression of GA metabolism/catabolism genes were readily detectable in the whole shoot samples (Figs. 4–7). Whether cryptochromes differentially regulate changes of GA_4 levels in specific cells and what molecular mechanism(s) may be responsible for such changes remain to be further investigated.

MATERIALS AND METHODS

Plant Materials

Arabidopsis (*Arabidopsis thaliana*) mutants *cry1*, *cry2*, *cry1cry2*, *phyA*, and *cry1cry2phyA* used in this study are in the Columbia background as described previously (Mockler et al., 2003).

To prepare an activation-tagging population, the *cry1cry2* plants were transformed using the binary vector pSKI015 and *Agrobacterium* strain GV3101 as described (Weigel et al., 2000). The T_1 seeds were harvested in pools and each pool contains seeds harvested from approximately 50 T_0 plants. T_1 seeds were germinated in white light and grown on compound soil, submerged in the herbicide Basta (approximately 0.006% ammonium glufosinate), and approximately 250,000 Basta-resistant T1 individuals were obtained. The herbicide-resistant individuals that showed hypocotyl length shorter than that of *cry1cry2* parent were selected as putative *scc-D* mutants, and they were separated from the rest of the mutant populations. Those *scc-D* lines that showed short hypocotyls in the T_2 generation grown in continuous blue light were subject to further genetic analysis. Genomic sequence flanking the T-DNA insert was identified using plasmid rescue (Weigel et al., 2000) or the Tail-PCR method (Liu et al., 1995). Hypocotyl lengths were measured manually for 6-d-old or 7-d-old seedlings (Lin et al., 1998). The sample size is larger than 20 seedlings, and the SDs are calculated.

To investigate the response of hypocotyl elongation to exogenous GA_3, paclobutrazol, or ancymidol, seeds were surface sterilized for 30 s in 70% ethanol, placed in 0.1% $Hgcl_2$ for 8 min, and rinsed five times with sterile, distilled water. About 100 seeds were placed in Murashige and Skoog agar growth medium. All hormone and inhibitor stocks were dissolved in 70% (V/V) ethanol at a concentration 500 times greater than the final concentration used. GA_3 (Shanghai Solvent), GA_4 (Sigma), and/or GA biosynthesis inhibitors ancymidol (Sigma) or paclobutrazol (J&K Chemical Ltd), were added into the Murashige and Skoog medium to the final concentrations indicated in the respective figures. Seeds were placed in the dark at 4°C for 4 d, exposed to white light for 12 h to enhance germination before transferring to temperature-controlled growth chambers, and grown under continuous blue, red, or FR light or in the dark at 22°C unless it is indicated otherwise (i.e. 26°C).

For studies of light- or clock-regulated gene expression, about 300 sterile seeds were sown on Murashige and Skoog agar medium, cold treated at 4°C for 4 d, exposed to white light for 12 h, and grown in the dark for 6 d before transfer to various light treatments. Alternatively, seedlings were grown under white light (20 ± 3 μmol m^{-2} s^{-1}) with long-day (16-h light/8-h dark) or short-day (8-h light/16-h dark) photoperiod for 10 d, and some petri dishes were transferred to continuous white light for 2 d. At the end of treatment, petri dishes were dipped in liquid nitrogen, and tissues (mostly shoot devoid of roots) were harvested by gentle scraping and stored at −80°C for RNA extraction.

Light Sources

In addition to light sources reported previously (Shalitin et al., 2002; Yu et al., 2007): LED-B (peak: 470 nm, half band width: 30 nm), LED-R (peak: 660 nm, half band width: 20 nm), and LED-FR (peak: 740 nm, half band width: 25 nm) were also used. Fluence rates of white, red, and blue light were measured using a Li-250 quantum photometer (LI-COR). The approximate fluence rates of FR light were estimated by plotting the relative fluence rates measured with a Li-250 quantum photometer to a near-linear standard curve of fluence rates measured with a spectroradiometer (T. Mockler and C. Lin, unpublished data).

GA Analysis

Whole shoot (all tissues except root) samples were used to analyze the GA content. For inductive light experiments, seeds were sown thickly on pots filled with potting mix, covered with a fine mesh, and placed in weak fluorescent light at 4°C for 4 d. Plants were transferred to dark at 22°C for 6 d and then transferred to 60 μmol m^{-2} s^{-1} blue light at 22°C using light source described (Platten et al., 2005).

For GA measurement, 3 to 4 g whole shoot samples were harvested, placed in ice-cold 80% methanol, homogenized, and the extract was filtered as described (Symons and Reid, 2003). Samples were concentrated to approximately 1 mL and loaded onto a preconditioned Sep-pak C_{18} cartridge in 0.4% acetic acid. GAs were eluted in 80% methanol in 0.4% acetic acid, dried, and fractionated using the reverse-phase C18 HPLC system (Jager et al., 2005). HPLC fractions corresponding to retention times of the relevant GAs were pooled, dried, methylated, and further purified by solvent partitioning as described (Jones et al., 2005). A total of 10 μL dry pyridine and 40 μL *N*,*O*-bis(trimethylsilyl) trifluoroacetamide were added to each vial and samples were heated at 80°C for 20 min. The samples were then dried before the addition of 15 μL *N*,*O*-bis(trimethylsilyl) trifluoroacetamide, incubated at 80°C for 15 min, dried under nitrogen gas, resuspended in 20 μL of chloroform, and analyzed by gas chromatography/tandem mass spectrometry (MS/MS) on a Varian 1200 triple quadrupole mass spectrometer (Jones et al., 2005). Injections of 1.5 μL were made in splitless mode at an injection temperature of 260°C onto a Varian VF5-ms column (30 m × 0.25 mm × 0.25 micron). The oven temperature was held at 50°C for 2 min, ramped to 230°C at 30°C per min, increased to 270°C at 5°C per min, and held at 270°C for 3 min. The transfer line temperature was 290°C, and the ion source was held at 220°C. The electron multiplier gain was 1,950 V. Carrier gas was helium at 1.2 mL per min. The MS was operated in selected reaction monitoring mode, with a Q1 peak width of 1.5 mass-to-charge ratio (m/z) units, and a Q3 peak width of 2.0 m/z units. The collision energy was −12 V, with the collision gas being argon at 1 mTorr. Prior to analyses instrument tuning was manually optimized in MS/MS mode rather than using the autotune feature. Based on preliminary full scan MS/MS experiments of standards, for endogenous GA_4 (methyl ester TMS ether) the selected precursor ion (Q1) was m/z 284 and the selected product ion (Q3) was m/z 224. For $^2H_2$ GA_4 (methyl ester TMS ether) the ions were 286 (Q1) and 226 (Q3). The amount of endogenous GA_4 was calculated from the peak areas. The internal standards ($[^2H_2]GA_4$, $[^2H_2]GA_{34}$, and $[^2H_2]GA_9$) were supplied by Professor L.N. Mander (Research School of Chemistry, Australian National University, Canberra).

Although the transient reduction in GA_4 level may result from reduced conversion of the precursor GA_9 to GA_4 and/or increased inactivation of GA_4 to the 2β-hydroxylated GA_{34}, we detected no significant changes in the GA_9 or GA_{34} levels. These discrepancies may be due to the fact that GA_9 level (less than 0.0025 ng g^{-1}) was below our detection limit, whereas GA_{34} levels were approximately 10-fold higher than that of GA_4, resulting in technical difficulties to detect relatively small or transient changes in the GA_{34} level under our experimental conditions. To compare GA content in etiolated seedlings with that grown in continuous blue light, 5-d-old whole shoot samples were used.

RNA Analyses

The accession numbers of genes discussed in this report are: *GA2ox1* (At1g78440), *GA2ox2* (At1g30040), *GA2ox3* (At2g34555, mRNA not detectable), *GA2ox4* (At1g47990), *GA2ox5* (pseudogene), *GA2ox6* (At1g02400), *GA2ox7* (At1g50960), *GA2ox8* (At4g21200), *GA20ox1* (At4g25420), *GA20ox2* (At5g51580), *GA20ox3* (At5g07200), *GA3ox1* (At1g15550), and *GA3ox2* (At1g80340).

Total RNA was isolated using Puprep RNAeasy mini kit (Ambiogen Life Tech Ltd). DNA-free RNA was obtained by RQ1 DNase I treatment according to the manufacturer's instructions (Promega). The amount of mRNA was analyzed using semiquantitative reverse transcription (RT)-PCR as decribed (Mockler et al., 2003). cDNA was prepared from 2 μg of total RNA by using Moloney murine leukemia virus reverse transcriptase according to the manufacturer's instructions (Promega). The cDNA was generally diluted 10-fold, and 1 μL of diluted cDNA was used in a 20 μL PCR reaction. DNA sequences of the PCR primers used in this study are the following: *ACT2*F (5′-CACTGTGCCAATCTACGAGGGT-3′), *ACT2*R (5′-CACAAACGAGGGCTGGAACAAG-3′); *GA2ox1F* (5′-CACTATCCACCATGTCCTCTTA-3′), *GA2ox1*R (5′-CAGACCAAGTAAACTCCTCGTA-3′); *GA2ox2*F (5′-AGAGGCGGAGAAGATGGTGAA-3′), *GA2ox2*R (5′-GACAAGGCATGGCAATGGTGC-3′); *GA2ox4*F (5′-CCGATCAATTCTTTGGTGAAG-3′), *GA2ox4*R (5′-AATGTTTGGTACAACCGTGGC-3′), *GA2ox6*F (5′-ATGATTACATACGCACGGTTAG-3′), *GA2ox6*R (5′-ACATACGTGGCTTCTTTGCTG-3′); *GA2ox7F* (5′-GGGAAACAAGTGAACGTGAGT-3′), *GA2ox7R* (5′-GAGAAACCTGGACAAGCCTAC-3′); *GA2ox8*F (5′-CGGAATCAGAGGCATTAGC-3′), *GA2ox8*R (5′-CCACCTTTGGGTTCGTCAT-3′); *A20ox1*F (5′-CAGCCATTTGGGAAGGTGTATC-3′), *GA20ox1*R (5′-CAAGCAGCTCTTGTATCTATCGT-3′); *GA20ox2*F (5′-TCAATATTGGTGACACTTTCAT-3′), *GA20ox2*R (5′-GATGGGATTGTTGTTGGTAATA-3′); *GA20ox3*F (5′-AAAATGGGCGATGGATACGAAG-3′), *GA20ox3*R (5′-CGAAAGCGTGAGGGTTAGGAG-3′), *GA3ox1*F (5′-CCGAAGGTTTCACCATCACTG-3′), *GA3ox1*R (5′-GAGGCGATTCAACGGGACTAAC-3′); *GA3ox2*F (5′-CCAGCCACCACCTCAAATAC-3′), *GA3ox2*R (5′-GTGAAGCACGCTCGGGAAGA-3′).

PCR was generally performed with a 5 min denaturation at 95°C followed by 24 to 35 cycles with each cycle composed of 95°C for 30 s, 55 to 60°C for 30 s, and 72°C for 30 s. PCR products were analyzed using 1.5% agarose gel eletrophoresis. RT-PCR reactions for each experiment were repeated at least three times, and the representative gel images were shown. The expression level of the *ACTIN2* gene was used as the internal control to normalize and calculate relative expression levels of genes tested using ImageJ (http://rsb.info.nih.gov/ij/).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At1g78440, At1g30040, At2g34555, At1g47990, At1g02400, At1g50960, At4g21200, At4g25420, At5g51580, At5g07200, At1g15550, and At1g80340.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Blue light-reduced mRNA expression of members of the *GA20ox* and *GA3ox* genes.

Supplemental Figure S2. The circadian rhythm of the expression of *GA20ox* genes.

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