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Cryptochrome and Phytochrome Cooperatively but Independently Reduce Active Gibberellin Content in Rice Seedlings under Light Irradiation

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In contrast to a wealth of knowledge about the photoregulation of gibberellin metabolism in dicots, that in monocots remains largely unclear. In this study, we found that a blue light signal triggers reduction of active gibberellin content in rice seedlings with simultaneous repression of two *gibberellin 20-oxidase* genes (*OsGA20ox2* and *OsGA20ox4*) and acute induction of four *gibberellin 2-oxidase* genes (*OsGA2ox4–OsGA2ox7*). For further examination of the regulation of these genes, we established a series of cryptochrome-deficient lines through reverse genetic screening from a *Tos17* mutant population and construction of knockdown lines based on an RNA interference technique. By using these lines and phytochrome mutants, we elucidated that cryptochrome 1 (cry1), consisting of two species in rice plants (cry1a and cry1b), is indispensable for robust induction of the *GA2ox* genes. On the other hand, repression of the *GA20ox* genes is mediated by phytochromes. In addition, we found that the phytochromes also mediate the repression of a *gibberellin 3-oxidase* gene (*OsGA3ox2*) in the light. These results imply that, in rice seedlings, phytochromes mediate the repression of gibberellin biosynthesis capacity, while cry1 mediates the induction of gibberellin inactivation capacity. The cry1 action was demonstrated to be dominant in the reduction of active gibberellin content, but, in rice seedlings, the cumulative effects of these independent actions reduced active gibberellin content in the light. This pathway design in which different types of photoreceptors independently but cooperatively regulate active gibberellin content is unique from the viewpoint of dicot research. This redundancy should provide robustness to the response in rice plants.

Keywords: Cryptochrome • Gibberellin • Leaf sheath elongation • Photomorphogenesis • Phytochrome • Rice • *Oryza sativa*.

Abbreviations: B, blue; cry, cryptochrome; FR, far-red; GA2ox, gibberellin 2-oxidase; GA20ox, gibberellin 20-oxidase; GA3ox, gibberellin 3-oxidase; PAC, paclobutrazol; phy, phytochrome; R, red; RNAi, RNA interference; RT–PCR, reverse transcription–PCR; WT, wild type.

Introduction

Rice is an important staple food that is eaten by billions of people worldwide (Hoshikawa 1989). To obtain an abundant harvest, formation of compact and sturdy seedlings is the first crucial task in the modern cultivation management of rice plants, which leads to robust growth in paddy fields at the vegetative stage. The shape of seedlings is influenced by various environment signals, including temperature, seeding density, soil moisture and light (Matsuo et al. 1995). The light signal has been demonstrated to be especially critical to the formation of compact seedlings through the drastic conversion of developmental programs from skotomorphogenesis to photomorphogenesis (Matsuo et al. 1995).

To monitor environmental light quality and quantity, plants are equipped with cryptochrome (cry), phytochrome (phy), phototropin and other photoreceptors (Lin 2002, Quail 2002, Demarsy and Fankhauser 2009). Cryptochrome is a blue/ultraviolet-A (B/UV-A) photoreceptor that is cognate with photolyase, a DNA repair enzyme (Cashmore et al. 1999). In angiosperms, cryptochrome genes form a small family. The Arabidopsis genome encodes at least two molecular species, cry1 and cry2, which display distinct properties. cry1 is a photostable species and is the major B light receptor for photomorphogenesis, while cry2 is photolabile (Lin et al. 1998). The cry2 species also mediates photomorphogenesis, but its action is limited to low-intensity B light (Lin et al. 1998). This species mainly mediates flowering time determination under natural

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conditions (Guo et al. 1998, El-Din El-Assal et al. 2001). We have identified three cryptochrome genes in the rice genome (Hirose et al. 2006). As phylogenetic investigation revealed that two of these genes encode a cry1-type receptor, they were named *OsCRY1a* (AB073546) and *OsCRY1b* (AB073547). The latter was categorized as cry2, named *OsCRY2* (AB103094). Our previous study using transgenic rice plants overexpressing one of the three species suggested that all of the cryptochrome species in rice plants mediate B light perception to suppress elongation of leaf sheaths and blades (Hirose et al. 2006).

Phytochrome is a red/far-red (R/FR) photoreceptor that mediates various responses in plants, such as light-dependent germination, photomorphogenesis, shade avoidance and photoperiodic control of flowering (Franklin and Quail 2010). In contrast to the prevailing view, phytochrome can perceive a broad range of light wavelengths. In addition to effective perception of R/FR light, inefficient but certain perception of B light can be recognized in several plant species (Shinomura et al. 1996, Xie et al. 2007). Phylogenetic investigations indicated that phytochrome also forms a small gene family, and the most primitive family in angiosperms consists of three members: phyA, phyB and phyC (Clack et al. 1994, Mathews et al. 1995, Alba et al. 2000). Arabidopsis and several other dicots have additive species derived from phyB, resulting in an Arabidopsis genome encoding five phytochrome genes: *PHYA*, *PHYB*, *PHYC*, *PHYD* and *PHYE* (Sharrock and Quail 1989, Clack et al. 1994). In contrast, monocots, especially rice, have kept the most primitive set, with only three genes (*PHYA*, *PHYB* and *PHYC*) in the genome (Kay et al. 1989, Dehesh et al. 1991, Tahir et al. 1998, Basu et al. 2000). We have isolated a complete set of rice phytochrome single mutants and constructed all combinations of the double and triple mutants (Takano et al. 2001, Takano et al. 2005, Takano et al. 2009). These mutants have already contributed not only to the elucidation of basic features of the rice phytochromes, but also to the identification of their roles in various photoresponses in rice plants (Ishikawa et al. 2005, Xie et al. 2007, Shimizu et al. 2009).

Gibberellin is a tetracyclic diterpene phytohormone that promotes several responses in plants, such as germination, stem elongation and flower induction (Yamaguchi 2008). Stem elongation in etiolated seedlings is the major action of gibberellin, and gibberellin activity rapidly decreases following light irradiation, which stops stem elongation in the seedlings (Symons and Reid 2003, Zhao et al. 2007). This correlation contributes to the formation of compact seedlings in the light. In addition, semi-dwarf cultivars of wheat and rice bred through the introduction of dwarfing traits achieve a spectacular increase in yield. This increase, called the 'Green Revolution', has enlarged the agriculturally sustainable population of the world (Hargrove and Cabanilla 1979). Recently, it was elucidated that these dwarfing traits were attributable to a partial deficiency of gibberellin biosynthesis (Sasaki et al. 2002). This conclusion has led to the use of gibberellin biosynthesis inhibitors to form compact seedlings in farming technology. In this way, elucidation of the molecular mechanisms regulating gibberellin metabolism is an important target for agricultural researchers.

Gibberellin is a group of substances possessing the *ent*-gibberellane skeleton. To date, >100 gibberellin species have been identified from fungal and plant tissues, although the number of biologically active gibberellin species is quite limited (Hedden and Kamiya 1997). Only GA_1, GA_3 and GA_4 have been confirmed to be endogenous active species in plants. Gibberellin is synthesized from *trans*-geranylgeranyl diphosphate through *ent*-kaurene (Hedden and Kamiya 1997, Olszewski et al. 2002, Yamaguchi 2008). A series of oxidations of *ent*-kaurene generates GA_{12}, the first gibberellin product in the biosynthesis pathway. Further steps in the pathway are divided into two branches, the non-13-hydroxylation and early-13-hydroxylation pathways. In rice plants in the vegetative stage, active gibberellin is synthesized through the early-13-hydroxylation pathway (Kobayashi et al. 1988). In this pathway, GA_{53} is converted into the biologically active GA_1 through a cascade of catalytic oxidations by two oxidases, gibberellin 20-oxidase (GA20ox) and gibberellin 3-oxidase (GA3ox). On the other hand, inactivation of gibberellin is also important for the regulation of gibberellin action. The most studied inactivation enzyme is gibberellin 2-oxidase (GA2ox), which catalyzes 2β-hydroxylation. This enzyme is recognized to be critical for the inactivation of gibberellin in various plants, especially during vegetative growth (Thomas et al. 1999).

Photoregulation of active gibberellin levels has been studied in several dicots, including lettuce (Toyomasu et al. 1992), pea (Reid et al. 2002) and Arabidopsis (Zhao et al. 2007). In pea seedlings during de-etiolation, a reduction of GA_1 triggered by light exposure could be observed with simultaneous down-regulation of *PsGA3ox1* and up-regulation of *PsGA2ox2* (Reid et al. 2002). Because available photoreceptor mutants in pea are limited, only the minor contribution of phyA could be clarified in this regulation (Reid et al. 2002), but the major photoreceptor remains unclear. Zhao et al. (2007) analyzed the B light-induced repression of gibberellin biosynthetic-related genes (*AtGA20ox1* and *AtGA3ox1*) and the expression of gibberellin inactivation-related genes (*AtGA2ox1*, *AtGA2ox2*, *AtGA2ox6* and *AtGA2ox8*) in a series of photoreceptor mutants of Arabidopsis. They detected a weak contribution of phyA to the induction of the *AtGA2ox1* gene, as in pea plants. In addition, they found that redundant actions of cry1 and cry2 mediate a large segment of the transcriptional regulation of these genes (Zhao et al. 2007). In contrast to the wealth of knowledge obtained from dicots, information about the photoregulation of gibberellin metabolism in monocots remains limited. In the present study, we focus on the photoregulation of gibberellin metabolism in rice seedlings. We found that a B light signal triggered a reduction of active gibberellin content with consistent changes in transcript levels of gibberellin biosynthesis- and inactivation-related genes. We utilized phytochrome-deficient mutants and newly established cryptochrome-deficient lines not only for identification of photoreceptors mediating the transcriptional regulations, but also for evaluation of each

photoreceptor in the regulation of active gibberellin content. Our results indicate that cry1 and phyB independently regulate different sets of gibberellin-related genes, but their cumulative effects cooperatively mediate reduction of the active gibberellin content in rice seedlings in the light.

Results

Gibberellin content in seedlings exposed to blue light

To evaluate the effect of B light irradiation on the regulation of gibberellin metabolism in rice seedlings, we measured the content of several molecular species of gibberellin in dark-grown seedlings before and after exposure to B light (10 μmol m^{-2} s^{-1}) for 24 h (**Fig. 1B**). Only GA_1 and GA_{20} showed significant reductions in their content after B light irradiation. GA_1 is a major active gibberellin species in rice (Kobayashi et al. 1988). GA_{20} is an inactive gibberellin species, but is the precursor molecule immediately before GA_1 in the biosynthesis pathway (**Fig. 1A**). On the other hand, the content of other gibberellin species was comparable between conditions. These results indicate that a B light signal triggers reductions of GA_1 and GA_{20} content in rice etiolated seedlings.

Expression of genes involved in biosynthesis, inactivation and signaling of gibberellin under B light irradiation

To approach the molecular mechanism of the B light response observed by the change in gibberellin content, we investigated the expression profiles for the genes encoding gibberellin biosynthetic enzymes GA20ox and GA3ox under B light irradiation. These genes generally form a small family. In rice plants, the *OsGA20ox* family consists of four members and the *OsGA3ox* family consists of two members (Toyomasu et al. 1997, Itoh et al. 2001, Sasaki et al. 2002, Sakamoto et al. 2004). **Fig. 2** and **Supplementary Fig. S1** show their expression profiles in etiolated seedlings exposed to B light. Among the members of the *OsGA20ox* family, *OsGA20ox2* and *OsGA20ox4* were down-regulated by B light irradiation. The lowest levels of these transcripts were estimated to be <5% of their initial values. On the other hand, *OsGA20ox1*, *OsGA20ox3*, *OsGA3ox1* and *OsGA3ox2* are probably irrelevant to the B light-induced regulation of gibberellin content because *OsGA20ox1* and *OsGA3ox2* were constitutively expressed even under B light irradiation (**Supplementary Fig. S1**). In addition, the expression levels of *OsGA20ox3* and *OsGA3ox1* were quite low under our experimental conditions (data not shown).

We next examined the expression of gibberellin inactivation-related genes in the *OsGA2ox* family. Ten *OsGA2ox* genes (*OsGA2ox1*–*OsGA2ox10*) have been identified in the rice genome (Sakamoto et al. 2004, Lee and Zeevaart 2005, Lo et al. 2008; **Supplementary Fig. S2**). A series of semi-quantitative reverse transcription–PCR (RT–PCR) experiments revealed that the expression of *OsGA2ox4*–*OsGA2ox7* genes was clearly induced by B light irradiation (**Fig. 2A, C**; **Supplementary Fig. S1**). The remaining members of the family are apparently not involved with the B light-induced changes in gibberellin content. Although the expression of *OsGA2ox1*, *OsGA2ox2*, *OsGA2ox3*, *OsGA2ox8* and *OsGA2ox9* could be observed, their levels were not affected by exposure to B light (**Supplementary Fig. S1**). Transcripts for *OsGA2ox10* could not be detected under our conditions (data not shown), implying that its expression had little influence on gibberellin content.

We also investigated the expression of genes involved in gibberellin signaling: *GID1* (Ueguchi-Tanaka et al. 2005), *GID2* (Sasaki et al. 2003) and *SLR1* (Ikeda et al. 2001). Their transcript levels were not influenced by B light irradiation (**Supplementary Fig. S1**), suggesting that gibberellin signaling is not controlled by B light, at least at their transcriptional level.

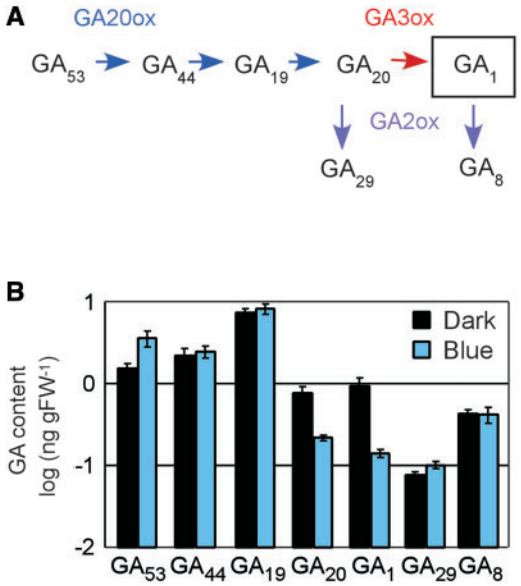

Fig. 1 Endogenous gibberellin content in dark-grown WT seedlings and their transitions after B light irradiation. (A) Schematic drawing of the metabolic flow of gibberellin species in the early-13-hydroxylation pathway. Blue, red and purple arrows indicate steps catalyzed by GA20ox (blue), GA3ox (red) and GA2ox (purple). (B) Endogenous content of several gibberellin species in 3-day-old WT etiolated seedlings (black bars) and 3-day-old etiolated seedlings exposed to B light (10 μmol m^{-2} s^{-1}) for another 24 h (blue bars). The contents of GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_1, GA_{29} and GA_8 are indicated by a logarithmic scale. All data are means of seven samples with standard errors.

Construction of cryptochrome-deficient lines

Mutants deficient in photoreceptors are important practically for identifying the roles of photoreceptors in various photoresponses. Because rice cryptochrome mutants had not been

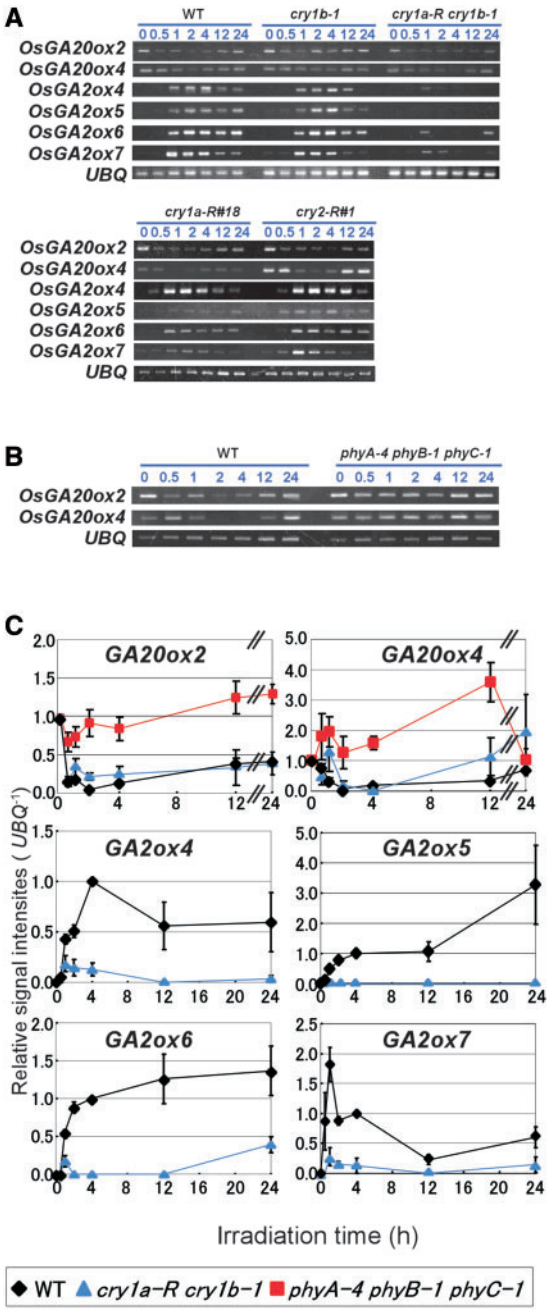

Fig. 2 The effect of B light on the expression of gibberellin biosynthesis- and inactivation-related genes. Three-day-old etiolated seedlings of WT, cryptochrome-deficient and phytochrome-deficient lines were exposed to B light (10 μmol m^{-2} s^{-1}) for the indicated periods. Transcripts of *UBQ* are shown as controls. (A) Expression of gibberellin biosynthesis- and inactivation-related genes in WT, *cry1b-1*, *cry1a-R cry1b-1*, *cry1a-R* and *cry2-R* lines. (B) Expression of *OsGA20ox2* and *OsGA20ox4* in WT seedlings and *phyA-4 phyB-1 phyC-1* mutants. (C) Relative transcript levels and their changes of *OsGA20ox2*, *OsGA20ox4* and *OsGA2ox4-7* in WT, *cry1a-R cry1b*, and *phyA-4 phyB-1 phyC-1* seedlings during B light irradiation.

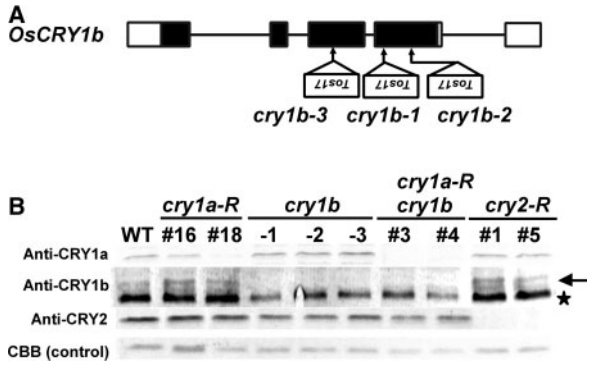

Fig. 3 Basic features of the cryptochrome mutants and knockdown lines used in this work are shown. (A) Schematic drawing of the *OsCRY1b* gene structure with the *Tos17* insertion sites of *cry1b* mutants isolated in this work. Introns (lines) and exons (boxes) are represented. Filled boxes indicate the coding regions, while open boxes correspond to 5′- and 3′-untranslated regions. (B) Western blotting analysis of OsCRY1a, OsCRY1b and OsCRY2 in *cry1b* mutants and cryptochrome-deficient transgenic lines. Proteins were extracted from 3-day-old etiolated seedlings of WT (Nipponbare), *cry1a-R* (#16 and #18), *cry1b-1*, *cry1b-2*, *cry1b-3*, *cry1a-R cry1b-1* (#3 and #4) and *cry2-R* (#1 and #5) lines. A 50 μg aliquot of protein was used for each sample analyzed. Coomassie brilliant blue-stained bands served as a loading control. The anti-CRY1b antiserum used in this study detected two bands on the blots. The upper band (marked with an arrow) corresponds to CRY1b. The lower band (marked with a star) was confirmed to be a cross-reactant between the antiserum and a protein other than CRY1b.

isolated previously, we attempted to isolate a series of them. In this work, we obtained three alleles of a *cry1b* mutant (*cry1b-1*, *-2* and *-3*) from a mutant population generated by insertion of the endogenous retrotransposon *Tos17* (Hirochika 1999). For each allele, a *Tos17* insertion was detected in the portion of the sequence corresponding to the third or fourth exon of *CRY1b* (**Fig. 3A**). We estimated the protein levels of the cryptochromes in these mutants by immunoblot analysis using specific antibodies raised against CRY1a, CRY1b or CRY2 (**Fig. 3B**). CRY1b disappeared from the immunoblots for proteins of these mutants. Moreover, no nascent bands could be detected on the blots (data not shown), indicating that the insertions of *Tos17* completely disrupt the *CRY1b* gene.

Despite our efforts, *cry1a* and *cry2* mutants could not be isolated from the mutant population; therefore, we constructed *CRY1a* and *CRY2* knockdown transgenic lines (named *cry1a-R* and *cry2-R*, respectively) using an RNA interference (RNAi) technique (Hannon 2002, Miki and Shimamoto 2004). **Fig. 3B** shows that CRY2 was not observable in *cry2-R* lines. In the *cry1a-R* lines, CRY1a was slightly detected on blots. In this study, *cry1a-R* #18 was preferentially used because the repression of *CRY1a* in this line was the tightest obtained to date. We also made cry1a/b doubly deficient lines by introducing the *cry1a-R* construct into the *cry1b-1* mutant (*cry1a-R cry1b-1*), because the lines are required for the definition of cry1

function under the mutually complementary relationship between cry1a and cry1b expected from their highly homologous amino acid sequences (Hirose et al. 2006, Zhang et al. 2006). **Fig. 3B** clearly shows that neither CRY1a nor CRY1b proteins could be detected in the *cry1a-R cry1b-1* seedlings.

Identification of photoreceptors involved in transcriptional regulation of *GA20ox* and *GA2ox* genes under B light irradiation

We measured the transcript levels of *GA20ox* and *GA2ox* genes in response to B light treatment in cryptochrome-deficient lines and compared them with those in wild-type (WT) seedlings. The B light-induced expression of *OsGA2ox4*–*OsGA2ox7* genes almost disappeared in *cry1a-R cry1b-1*, but remained unchanged in *cry1a-R* and *cry1b-1* (**Fig. 2A, C**).

On the other hand, the repression of *OsGA20ox2* and *OsGA20ox4* by the B light treatment could be observed not only in the single crytochrome-deficient lines (*cry1a-R*, *cry1b-1* and *cry2-R*), but also in the cry1-deficient line (*cry1a-R cry1b-1*), suggesting that photoreceptors other than cryptochromes may mediate the repression of these genes (**Fig. 2A, C**). We attempted to examine the involvement of phytochromes in this repression. As expected, the transient repression of the genes could not be detected in the phytochrome triple mutant (*phyA-4 phyB-1 phyC-1*; **Fig. 2B**).

Expression of genes involved in biosynthesis, inactivation and signaling of gibberellin under R light irradiation

To dissect the contribution of phytochrome to the regulation of gibberellin-related gene expression, we repeated semi-quantitative RT–PCR analyses for the estimation of a series of gibberellin-related transcripts under R light irradiation. We detected down-regulation of the *OsGA20ox2* and *OsGA20ox4* genes in WT seedlings during R light irradiation with almost identical kinetics to those induced by B light irradiation (**Figs. 2**, **4**; **Supplementary Fig. S1**). This repression was significantly diminished in the *phyB* mutant and completely disappeared in the *phyA phyB* mutant (**Fig. 4A, B**). In contrast, nearly comparable down-regulation of these genes could be detected in the *phyA*, *phyC* and *phyA phyC* mutants (**Fig. 4A**). These results indicate that phyB is the major photoreceptor for the repression of *OsGA20ox2* and *OsGA20ox4*, while phyA contributes to the regulation, but its action is supplemental and then appears only under the phyB-deficient background. The R light experiments revealed that the transcription of *OsGA3ox2*, which was not affected by B light irradiation, was repressed by R light irradiation. This repression was also mediated by phyB with a minor contribution from phyA (**Fig. 4A, B**; **Supplementary Fig. S1**). The most probable reason why this gene could not respond to the B light signal is that the P_{FR}/P_R ratio in the photoequilibrium state given under the B light signal might be too low to induce the response clearly.

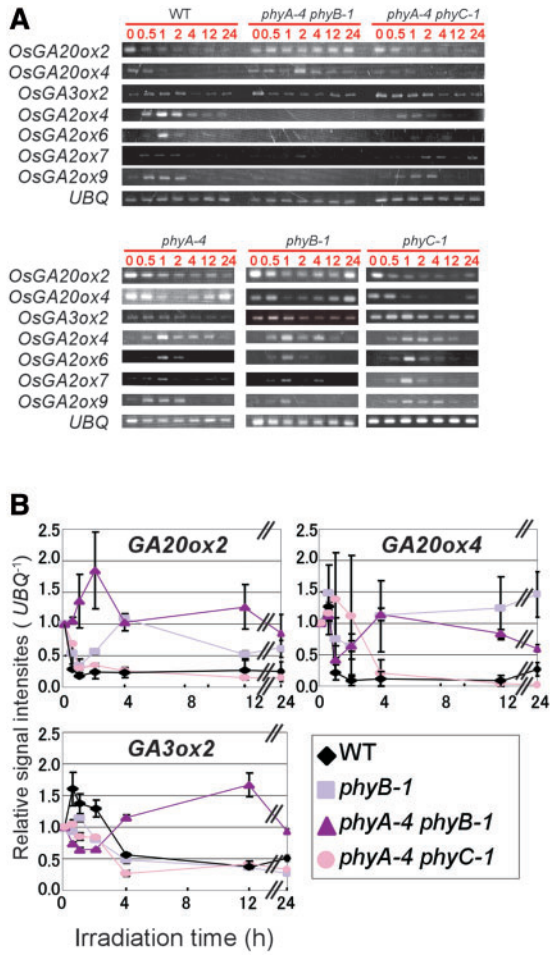

Fig. 4 The R light-induced modulation of the expression of gibberellin biosynthesis- and inactivation-related genes in WT seedlings and various phytochrome mutants. Three-day-old etiolated seedlings of WT and phytochrome mutants were exposed to R light (10 μmol m^{-2} s^{-1}) for the indicated period. Transcripts of *UBQ* are shown as controls. (A) The expression of gibberellin biosynthesis- and inactivation-related genes in WT, *phyA-4 phyB-1*, *phyA-4 phyC-1*, *phyA-4*, *phyB-1* and *phyC-1* lines during R light irradiation was examined. (B) Relative transcript levels of *OsGA20ox2*, *OsGA20ox4* and *OsGA3ox2* in WT, *phyB-1*, *phyA-4 phyB-1* and *phyA-4 phyC-1* were estimated from gel images of RT–PCR assays.

A series of R light experiments elucidated another minor effect of phytochromes on the light-induced expression of *OsGA2ox* genes. The B light induction of the *OsGA2ox4*, *OsGA2ox6* and *OsGA2ox7* genes was almost eliminated in the *cry1a-R cry1b-1* line (**Fig. 2A, C**); however, weak transient induction remained. This residual induction might be attributable to a phytochrome action, because the weak induction could be reproduced by R light irradiation with similar kinetics to those induced by B light irradiation (**Fig. 4A**; **Supplementary Fig. S1**). In addition, the R light-mediated induction disappeared in the *phyA phyB* mutant (**Fig. 4A**).

Gibberellin content in cry1- or phytochrome-deficient seedlings under light conditions

In the previous sections, we elucidated the transcriptional regulation of genes encoding gibberellin biosynthesis and inactivation enzymes under light treatments. We also revealed the involvement of cryptochromes and phytochromes in this regulation. We next examined the direct consequences of photoreceptor deficiency on the reduction of active gibberellin content in the light. We measured the content of GA_{20} and GA_1 in dark-grown WT, *cry1a-R cry1b-1* and *phyA-4 phyB-1* seedlings before and after light treatments (**Fig. 5A, B**). This figure has an unsolved problem in that GA_1 content in the dark-grown *cry1a-R cry1b-1* seedlings was slightly high compared with those of the WT and *phyA-4 phyB*-1 seedlings. We considered that the elevated GA_1 level was likely to be a consequence of a somatic variation introduced through construction of the transgenic line. We supposed that values for the GA_1 content in the *cry1a-R cry1b-1* seedlings were elevated to a similar extent.

Fig. 5 displays the predominant implication of cry1 in the regulation of gibberellin metabolism. A drastic reduction of GA_{20} levels by B light treatment in WT seedlings completely disappeared in *cry1a-R cry1b-1* seedlings. A similar reduction of GA_1 content was significantly moderated in the cry1-deficient line. They were thought to be effects of cry1-mediated induction of *GA2ox* genes (**Fig. 2A, C**), which should increase GA2ox activity and then accelerate to reduce both GA_1 and GA_{20} content through catalytic oxidation. If so, the drastic reduction of the GA_1 and GA_{20} levels that was completely conserved even in the *phyA-4 phyB-1* seedlings indicates the predominance of cry1 in the B light-induced reduction of the active gibberellin content in the seedlings. However, in parallel, we could recognize the substantial contribution of phytochromes to the regulation, which could be detected in observations that the GA_1 content was reduced by R light irradiation in WT seedlings and the reduction completely disappeared in *phyA-4 phyB-1* seedlings. In addition, moderate reduction of the GA_1 content in *cry1a-R cry1b-1* seedlings after B light irradiation might be attributed to the action of phytochrome. The phytochrome signal is likely to reduce only the GA_1 content, which should result in the signal decreasing the biosynthesis of GA_1 through the repression of *GA20ox* and *GA3ox* genes (**Figs. 2, 4**).

Lengths of leaf sheaths under B light irradiation

In this work, we revealed that cryptochromes and phytochromes mediate the reduction of active gibberellin content in rice seedlings, probably through transcriptional regulation of a limited number of gibberellin-related genes. These features might influence the morphological phenotypes of the seedlings, because gibberellin is a pivotal phytohormone for promoting stem elongation in rice plants. Therefore, we measured the lengths of the second leaf sheaths of the cryptochrome-deficient lines and WT seedlings grown under various fluence

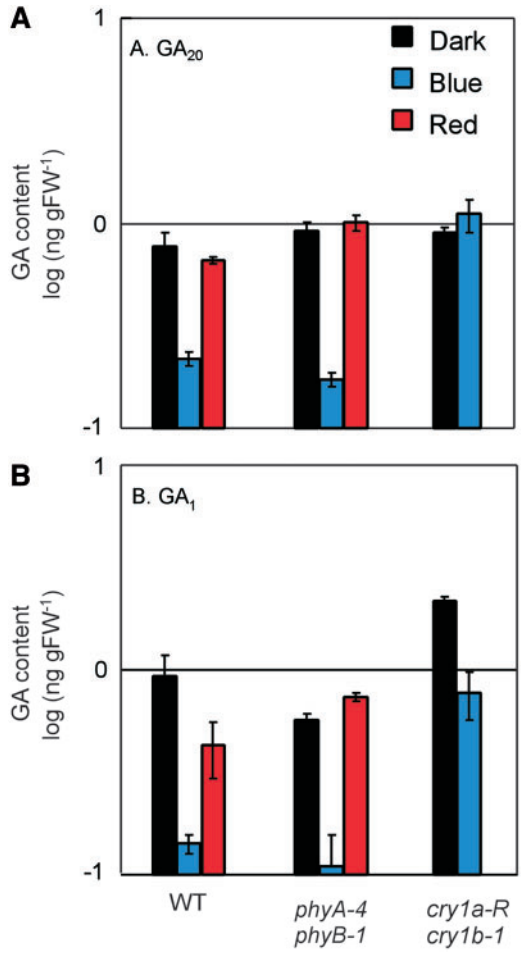

Fig. 5 The endogenous content of GA_{20} (A) and GA_1 (B) in 3-day-old dark-grown seedlings of *cry1a-R cry1b-1*, *phyA-4 phyB-1* and WT lines and their transitions after exposure to B or R light for another 24 h are shown by a logarithmic scale. Black bars indicate their content in dark-grown seedlings, while red and blue bars represent their content after R or B light irradiation for another 24 h, respectively. All data are means estimated from at least three samples. Error bars indicate the SE of the data.

rates of B light and examined their fluence rate responsiveness (**Supplementary Fig. S3B**). In this figure, we do not show the dark control values, because we could not measure them from the dark-grown seedlings. A significant number of these seedlings displayed a dark phenotype in which elongation of the leaves and leaf sheaths was severely restricted and these parts could not grow from the coleoptiles.

The lengths of the second leaf sheaths of WT seedlings reflected obvious fluence rate-dependent shortening. The lengths of *cry1a-R* and *cry1b-1* seedlings showed similar responses, but these values were slightly higher than those of WT seedlings under all fluence rates examined (**Supplementary Fig. S3B**). The lengths of *cry2-R* seedlings were longer than those of WT seedlings under weak B light; however, the tendency disappeared under intense B light. Under 30 $\mu mol\ m^{-2}\ s^{-1}$

B light irradiation, the lengths of the second leaf sheaths of *cry2-R* were almost equivalent with those of WT seedlings (**Supplementary Fig. S3B**). In *cry1a-R cry1b-1* seedlings, these lengths were significantly longer than those of WT seedlings, especially under a high fluence rate ($\geq$10 μmol m^{-2} s^{-1}) (**Supplementary Fig. S3A, B**). The result indicates that cry1 deficiency severely weakened the fluence rate-dependent shortening of leaf sheaths. However, it is noteworthy that the cry1-deficient line never lost its responsiveness, because the fluence rate response curve of the line was slightly downward in the graph (**Supplementary Fig. S3B**).

Effects of exogenous GA_1 on elongation of the second leaf sheath under B light irradiation

In earlier sections, we demonstrated the predominance of cry1a/b actions in the B light-induced reduction of active gibberellin content in rice seedlings, which should be a consequence of rapid induction of the *OsGA2ox4–OsGA2ox7* genes triggered by the B light irradiation. Robust induction of GA2ox enzymes can rapidly remove biologically active gibberellin from the cells, which implies that it is connected to quick suppression of leaf sheath elongation. We attempted to evaluate GA2ox activity in the leaf sheath cells through the effectiveness of exogenous GA_1 on the elongation of second leaf sheaths. To remove any influences of endogenous active gibberellin from the assay, we added a gibberellin biosynthesis inhibitor, paclobutrazol (PAC), to the agar medium. PAC has been reported to inhibit *ent*-kaurene oxidase and consequently cause a drop in content of almost all molecular species of gibberellin (Hallahan et al. 1988). Even in the presence of PAC, leaf sheaths could elongate up to approximately 2.7 mm, which was probably due to gibberellin-independent elongation. The addition of PAC strongly inhibited further elongation of the second leaf sheaths of the *cry1a-R cry1b-1* seedlings when grown under B light irradiation for 8 d (**Fig. 6C**), indicating that de novo synthesis of gibberellin is essential for the further elongation of leaf sheaths in this mutant. Next, we investigated the effect of exogenous GA_1 on the elongation of the second leaf sheaths in WT seedlings and in *cry1a-R cry1b-1* seedlings under B light irradiation in the presence of PAC. The second leaf sheaths of the GA_1-treated *cry1a-R cry1b-1* seedlings were significantly longer than those of the seedlings without GA_1, while GA_1 application had a weak effect on WT seedlings (**Fig. 6B, C**). The difference in effectiveness against the exogenous GA_1 in WT seedlings and the *cry1a-R cry1b-1* line implies two possibilities; one is that the cryptochrome mutations affect responsiveness to GA_1, and the other is that the activity of a GA_1-inactivating enzyme, probably GA2ox, is higher in WT seedlings than in *cry1a-R cry1b-1* seedlings. The latter possibility is more probable because it correlates with our observations that WT seedlings under B light irradiation displayed robust induction of *GA2ox* genes, while the mutant lacked their induction (**Fig. 2A, C**).

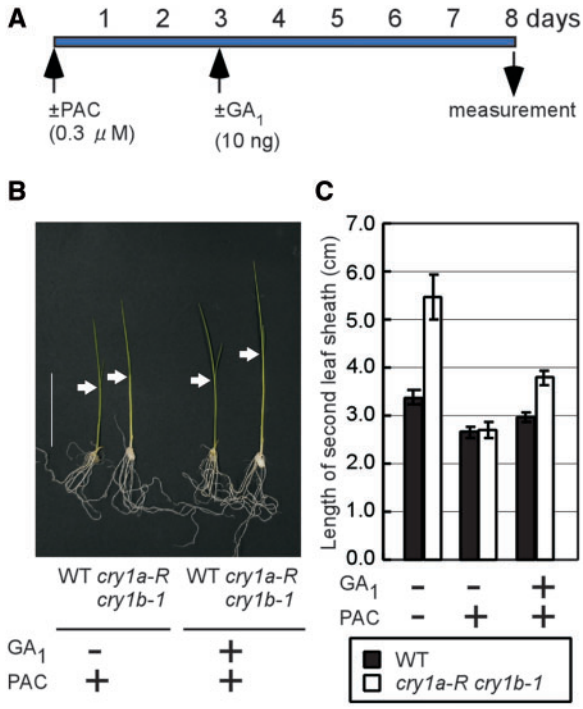

Fig. 6 The effect of exogenous GA_1 treatment on the suppression of leaf sheath elongation in the cry1-deficient line. (A) Schematic drawing of the experimental timetable for PAC and GA_1 treatments. (B) This photograph shows typical seedlings grown under B light (10 μmol m^{-2} s^{-1}) for 8 d at 28°C in the presence of PAC (0.3 μM) with and without GA_1. Arrows indicate the lamina joints, a monocotyledonous structure between a leaf sheath and a leaf blade. Scale bar = 30 mm. (C) The lengths of second leaf sheaths in WT and *cry1a-R cry1b-1* seedlings under B light with or without (+ or −) exogenous GA_1 treatment in the presence or absence (+ or −) of PAC. Bars represent the lengths of second leaf sheaths of WT (black) and *cry1a-R cry1b-1* (white) lines (n = 3–6).

Discussion

We started this work from measurements of endogenous gibberellin content in dark-grown seedlings before and after B light irradiation for 24 h (**Fig. 1B**). In our assays, the gibberellin species in the early-13-hydroxylation pathway could be detected at relatively high levels. On the other hand, the content of the gibberellin species in the non-13-hydroxylation pathway was relatively low. In particular, GA_9, GA_4 and GA_{34} could not be detected in our samples (data not shown). This is consistent with past reports that the early-13-hydroxylation pathway is predominant for gibberellin biosynthesis in rice seedlings during vegetative growth (Kobayashi et al. 1988). **Fig. 1B** displays the content of molecular species in the pathway, showing that the levels of GA_1 and GA_{20} dropped significantly after B light irradiation. This result indicates that rice plants possess mechanisms to reduce the content of the biologically active gibberellin species GA_1 along with that of its cognate precursor (GA_{20}) after perception of B light signals.

To understand how B light stimuli decrease active gibberellin content in rice plants, we examined the transcript levels of *GA20ox* and *GA3ox* as gibberellin biosynthesis-related genes and those of *GA2ox* as gibberellin inactivation-related genes, because correlations between their transcript levels and active GA content have been reported in several plant species (Reid et al. 2002, Zhao et al. 2007). **Fig. 2A** and **C** and **Supplementary Fig. S1** show that B light led to the repression of *OsGA20ox2* and *OsGA20ox4* and the induction of *OsGA2ox4–OsGA2ox7*, while the expression of other gibberellin biosynthesis- and inactivation-related genes was not affected. In addition, the gibberellin signaling-related genes *GID1*, *GID2* and *SLR1* did not respond to the B light. Thus, in rice plants, the transcript levels for limited members of the gibberellin biosynthesis- and inactivation-related enzymes were regulated by B light irradiation. These changes in their transcript levels are likely to be connected to the observed reductions of GA_1 and GA_{20} after B light irradiation (**Fig. 1B**).

The application of cryptochrome-deficient lines and phytochrome mutants to the analyses of transcriptional profiles of the gibberellin-related genes opened up consideration of the functions of each photoreceptor in the regulation. For example, the induction of *OsGA2ox4–OsGA2ox7* genes by B light irradiation disappeared in *cry1a-R cry1b-1* seedlings, but remained unchanged in lines deficient in one of the cry1 species (*cry1a-R* and *cry1b-1*; **Fig. 2A, C**), indicating that cry1a and cry1b complementarily mediate the induction of *OsGA2ox4–OsGA2ox7* genes. This feature is consistent with a wealth of past observations in dicots (Zhao et al. 2007). On the other hand, the repression of *OsGA20ox2* and *OsGA20ox4* under B light irradiation was not influenced by a deficiency of any cryptochromes (**Fig. 2A, C**). Surprisingly, the repression disappeared in phytochrome triple mutants (**Fig. 2B, C**), which indicates that the repression is mediated by phytochromes rather than cryptochromes. It is not unusual that rice phytochrome can conduct various B light responses, especially in juvenile seedlings (Takano et al. 2005, Xie et al. 2007). However, this phytochrome-dependent regulation of the *GA20ox* genes is unique from the viewpoint of past observations from Arabidopsis studies. In Arabidopsis, the *GA20ox* genes are also repressed by B light signals, but this repression is mediated by the cooperation of cry1 and cry2 (Zhao et al. 2007). A slight phyA contribution could be observed in the repression, but it was shown to be very weak. Furthermore, there has been no report suggesting a phyB contribution to the repression. Arabidopsis phyB was reported to control gibberellin biosynthesis-related genes (*AtGA3ox1* and *AtGA3ox2*) and inactivation-related genes (*AtGA2ox2*), but the regulation was observed in seed germination (Yamaguchi et al. 1998, Oh et al. 2006). In addition, phyB reportedly mediated the induction of *GA3ox1* and *GA3ox2* expression and the reduction of *GA2ox2* expression under light irradiation, which is in contrast to our observations.

A series of B light experiments indicated the unique contribution of phytochromes in the repression of *OsGA20ox* genes in rice plants. If this is true, experiments using R light stimuli should emphasize the contribution; therefore, we examined and compared the expression of gibberellin biosynthesis- and inactivation-related genes under R light irradiation in WT seedlings and a set of phytochrome mutants (**Fig. 4A, B**; **Supplementary Fig. S1**). The R light irradiation of the WT seedlings led to repression of the gibberellin biosynthetic genes *OsGA20ox2* and *OsGA20ox4* with similar kinetics to those induced by B light irradiation. In addition, the R light irradiation also repressed *OsGA3ox2* gene expression. Further examination showed another minor R light response as a transient induction of gibberellin inactivation-related genes (*GA2ox4*, *Ga2ox6*, *GA2ox7* and *GA2ox9*). Analyses using a series of phytochrome mutants revealed that all of these R light responses were mediated by phyB with the synergistic support of phyA (**Fig. 4A, B**; **Supplementary Fig. S1**). These results can explain a phenotype with phyB-dependent suppression of leaf sheath elongation under R light (Takano et al. 2005). These results suggest that phyB practically mediates the regulation of gibberellin metabolism in rice plants. This feature is different from that in Arabidopsis, in which phyB tends to regulate gibberellin responsiveness rather than gibberellin metabolism during de-etiolation (Reed et al. 1996).

Our next efforts focused on evaluating photoreceptors in the regulation of active gibberellin content. We measured the content of GA_1 and GA_{20} in WT, cry1-deficient (*cry1a-R cry1b-1*) and phytochrome double mutant (*phyA-4 phyB-1*) etiolated seedlings before and after light irradiation. In these measurements, the GA_1 content in the dark-grown seedlings of *cry1a-R cry1b-1*, which is the control value for the line, was slightly high compared with those of WT and *phyA-4 phyB-1* seedlings. The elevated GA_1 level was likely to be a consequence of an abnormal mesocotyl elongation phenotype of the *cry1a-R cry1b-1* seedlings grown in darkness, which was probably due to a somatic variation introduced through construction of the transgenic line. We could not determine the real reason for the results of the phenotype, but we thought that the basic photoresponse of GA_1 content would not be influenced even with the abnormal phenotype.

These measurements revealed that B light effectively reduced both GA_{20} and GA_1 content in the WT seedlings, which could be interpreted to reflect that the changes were attributed to the cumulative effects of the cry1-mediated induction of the *GA2ox4-7* genes and the phytochrome-mediated repression of the *GA20ox2* and *GA20ox4* genes. On the other hand, R light irradiation moderately reduced only the GA_1 content, possibly because R light irradiation repressed the expression of the biosynthesis-related genes *GA20ox* and *GA3ox*, which can reduce GA_1 biosynthesis, but cannot consume previously synthesized GA_1. Certainly, weak induction of *GA2ox4*, *GA2ox6*, *GA20x7* and *GA2ox9* by R light was detected, but it should not be enough for complete consumption of the previously synthesized GA_1. Similar moderate reduction of the GA_1 content could be observed in the cry1-deficient line under

B light irradiation, which was probably due to the action of phytochromes.

The B light-irradiated phytochrome mutant showed drastic reductions in GA_1 and GA_{20} content. This reduction could not be distinguished from that observed in WT seedlings, suggesting that cry1 action is predominant in the photoregulation of active gibberellin content. In summary, the cry1-dependent reduction of active gibberellin content, which is probably caused by the induction of *GA2ox4–GA2ox7* genes, is more important for reducing the gibberellin content in this condition. However, our results simultaneously displayed the presence of a phytochrome-dependent mechanism for the regulation of GA_1 content in rice plants.

In dicots, B light-induced suppression of stem elongation is considered in relation to the photoregulation of gibberellin metabolism (Reid et al. 2002, Zhao et al. 2007). Therefore, B light-induced reduction of active gibberellin content in rice seedlings should affect morphological phenotypes. In particular, the lengths of the leaf sheaths should be influenced by the changes in gibberellin content. We next examined the fluence rate dependence of the lengths of second leaf sheaths under B light irradiation using WT and various cryptochrome-deficient lines (**Supplementary Fig. S3**). The results suggest that cry2 mediates the suppression of the elongation of leaf sheaths under B light with fluence rates $<10\ \mu mol\ m^{-2}\ s^{-1}$, while cry1 can conduct the response over a wide range of fluence rates. In particular, cry1 preferentially transmits strong B light at fluence rates $>10\ \mu mol\ m^{-2}\ s^{-1}$. Similar fluence rate preferences have been reported for Arabidopsis cryptochromes (Lin et al. 1998), suggesting that each molecular species shares basic features with their orthologs. The fluence rate responses also indicated that weak responsiveness to B light was retained even in the *cry1a-R cry1b-1* seedlings. The retained responsiveness might be attributed to the action of phytochromes.

In this study, we showed that five members of the *OsGA2ox* gene family (*GA2ox4–GAox7* and *GA2ox9*) are photoregulated by cryptochromes, phytochromes or both (**Figs. 4A**, **5A**), which probably contributes to the reduction of active gibberellin content under light irradiation. GA2ox members are subdivided into three classes (class I, II and III) based on phylogenetic relationships (**Supplementary Fig. S2**; Schomburg et al. 2003, Lee and Zeevaart 2005). OsGA2ox4 and OsGA2ox7 belong to class I, while OsGA2ox5, OsGA2ox6 and OsGA2ox9 are categorized into class III. We could not find any class II members that were under photoregulation. Recently, it has been reported that members of each class have individual substrate preferences in terms of the backbone structure of gibberellin (Schomburg et al. 2003, Lee and Zeevaart 2005). In the gibberellin biosynthesis pathway, almost all precursor species consist of 20 carbon atoms (C_{20}-gibberellin). GA20ox converts a methyl group at the C-20 position of gibberellin to an aldehyde by sequential oxidations and finally removes this carbon atom, which produces a gibberellin species with 19 carbon atoms (C_{19}-gibberellin). Therefore, products of GA20ox and their further metabolites, such as GA_1, GA_{20}, GA_{29} and GA_8, are the C_{19}-gibberellins (Olszewski et al. 2002, Yamaguchi 2008). Members of class I and II predominantly catalyze C_{19}-gibberellins including active gibberellin species and close cognate precursors. In rice seedlings, the class I and II members seem to convert GA_{20} and GA_1 into their respective inactive species, GA_{29} and GA_8. In contrast, the class III members were able to catalyze only C_{20}-gibberellin species, which are the upstream precursor species, namely GA_{53}, GA_{19} and GA_{44}, in the early-13-hydroxylation pathway. Therefore, the class III enzymes convert precursors into their metabolites to prevent their activation. In short, rice plants are equipped with two types of photoregulated GA2ox enzymes, one of which reduces active gibberellin content directly and the other eliminates precursor species under light irradiation.

We further investigated the importance of B light induction of class I *GA2ox* genes in the suppression of leaf sheath elongation through a comparison of the effectiveness of exogenous GA_1 in WT and *cry1a-R cry1b-1* seedlings (**Fig. 6B, C**). The effectiveness is probably correlated with the total activities of class I and II GA2ox. Exogenous GA_1 induced the elongation of leaf sheaths in *cry1a-R cry1b-1* seedlings, but not in WT seedlings (**Fig. 6B, C**), suggesting that the WT seedlings could eliminate exogenous active gibberellin species efficiently. This result is consistent with the fact that WT seedlings showed robust *GA2ox* expression (**Fig. 2A**). On the other hand, the cry1-deficient seedlings might not eliminate exogenous active gibberellin species, which could be attributable to a deficiency of induction of the *OsGA2ox4* and *OsGA2ox7* genes by B light irradiation (**Fig. 2A**). These results also suggest that the induction of class I *OsGA2ox* genes under B light irradiation is important for the suppression of leaf sheath elongation in the light.

In summary, the results obtained from our experiments using monochromatic light sources and a series of photoreceptor mutants supply an opportunity to consider the mechanism that regulates gibberellin metabolism in natural light conditions (**Fig. 7**). Solar radiation, which consists of a broad range of wavelengths of visible light, can trigger two types of photoreceptors involved in the regulation, namely phytochromes and cryptochromes. Phytochromes mediate the repression of several members of the *GA20ox* and *GA3ox* gene families, which is connected to a reduction in the biosynthesis of GA_1. In parallel, cryptochrome signals induce several *GA2ox* genes. GA2ox enzymes decrease active gibberellin levels through two processes: the reduction of precursor gibberellin species by class III enzymes and the reduction of active gibberellin species directly by class I enzymes. The cumulative effects of these independent actions are probably to decrease the GA_1 content in rice seedlings, which should consequently suppress the elongation of leaf sheaths and blades and lead to the formation of compact seedlings. This pathway design, in which two different types of photoreceptors mediate the reduction of active gibberellin content independently, is significantly different from that in Arabidopsis. The redundant pathways provide

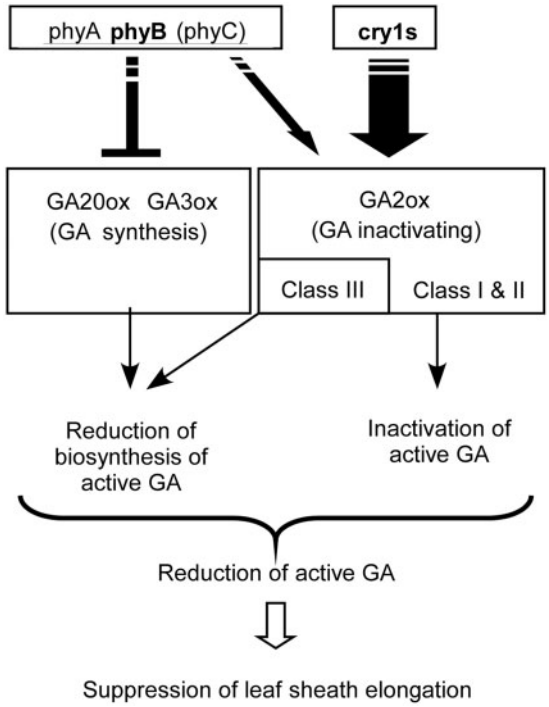

Fig. 7 A model for the photoregulation of gibberellin content in rice seedlings. The cry1 (cry1a/b)-mediated rapid induction of a limited number of the *OsGA2ox* gene family members (*OsGA2ox4*–*OsGA2ox7*). Phytochromes mediated the repression of gibberellin biosynthesis-related genes (*OsGA20ox2*, *OsGA20ox4* and *OsGA3ox2*) and weak induction of gibberellin inactivation-related genes (*OsGA2ox4*, *OsGA2ox6*, *OsGA2ox7* and *OsGA2ox9*). These independent actions cumulatively mediate a reduction in active gibberellin content in the cells, which is probably connected to the suppression of leaf sheath elongation in rice seedlings under the light.

robustness to the network in rice plants. Further research is required to clarify whether the individual pathway design reported here is specific to rice or is widely distributed in monocots.

Materials and Methods

Plant materials and growth conditions

We used the cultivar Nipponbare as the WT strain in this study. In addition, the rice cryptochrome-deficient lines newly established in this study and a series of phytochrome mutants (*phyA-4*, *phyB-1*, *phyC-1*, *phyA-4 phyB-1*, *phyA-4 phyC-1* and *phyA-4 phyB-1 phyC-1*; Takano et al. 2001, Takano et al. 2005, Takano et al. 2009) were used. Dehusked seeds were surface-sterilized in liquid sodium hypochlorite, sown onto 0.5% (w/v) agar and grown at 28°C under the various light conditions mentioned in the text. An R light-emitting diode panel (Model LED-R, EYELA) and a B light-emitting diode panel (Model LED-B, EYELA) were used as monochromatic light sources.

Measurement of gibberellin content

Rice seedlings grown in the dark for 3 d or in the dark for 3 d with 24 h B or R light irradiation were harvested and the samples were kept at −80°C until gibberellin extraction. Gibberellin levels were determined by liquid chromatography-selected reaction monitoring on a quadrupole/time-of-flight tandem mass spectrometer (Q-Tof Premier, Waters) connected to an Acquity Ultra Performance liquid chromatograph equipped with a reverse phase column (Acquity UPLC BEH-C18, Waters) as described previously (Varbanova et al. 2007). We used ^{2}H-labeled gibberellin species as internal standards. The labeled gibberellin species were purchased from Professor Lewis Mander (Australian National University, Canberra, Australia).

RT–PCR

Total RNA was isolated from aerial parts of the seedlings using an RNeasy Plant Mini kit (QIAGEN). To remove any genomic DNA contamination, the RNA samples were treated with RNase-free DNase I (QIAGEN) according to the manufacturer's instructions. A 1 μg aliquot of total RNA was used as a template to synthesize cDNA using a ReverTra Ace kit (TOYOBO). Gibberellin-related genes were amplified from the cDNA using gene-specific primers (see **Supplementary Table S2**). In these reactions, 1 μl of cDNA products was added to 20 μl of PCR mixture and the total number of amplification cycles was adjusted to keep the results semi-quantitative. After PCR, the products were examined by agarose gel electrophoresis with ethidium bromide staining. At least three biological repetitions of RT–PCR assays were carried out to maintain statistical reliability. Gel images were obtained with the AlphaImager Mini System (ProteinSimple) and the relative amounts of transcripts were estimated from the gel images using ImageJ software (http://rsb.info.nih.gov/ij/). Levels of *OsUBQ* (D12629) transcript were stable during our experimental conditions (data not shown); therefore, we used this transcript as the quantitative standard. Representative gel images of the RT–PCR analyses are shown in each figure.

Screening of cryptochrome mutants

The *cry1b* mutants were isolated from a mutant population of rice (*Oryza sativa* cv. Nipponbare) generated by random insertion of an endogenous retrotransposon, *Tos17* (Hirochika 1999), which is available from the Rice Genome Resource Center (RGRC) of the National Institute of Agrobiological Sciences (Tsukuba, Japan, http://www.rgrc.dna.affrc.go.jp/index.html). The first screening was carried out by PCR against three-dimensional panels of mutant DNA pools supplied from the RGRC using primer sets designed for the amplification of a *Tos17*-tagged *CRY1b* gene. To eliminate false amplifications, nested PCR was conducted against PCR products obtained in the first screening. Primer sequences used in the screening are listed in **Supplementary Table S1**. The amplified DNA fragments were cloned and sequenced to confirm the insertion of *Tos17* into the *CRY1b* gene. The matrix of appearance of PCR

products on three-dimensional panels enabled us to identify candidate lines in the mutant population. Consequently, three alleles having a *cry1b* mutation were screened from the population. M_2 seeds of the lines supplied from the RGRC were cultivated for the establishment of *cry1b* homozygous lines (*cry1b-1*, *-2* and *-3*). Isolation of *cry1a* and *cry2* mutants was attempted in the same manner, but they could not be obtained from this population.

Construction of transgenic plants

To construct *CRY1a* and *CRY2* knockdown transgenic lines using an RNAi technique (Miki and Shimamoto 2004), each unique sequence of these genes was amplified by PCR from each cDNA clone isolated in our previous work (Hirose et al. 2006). The sequences of primer pairs for amplification of *CRY1a* were 5′-CACCTTTCAGACTACAATTCACCGGG-3′ and 5′-CAC TTGATTCATGCACACCAAG-3′, and those for *CRY2* were 5′-C ACCAGATGGTGAGGTTGTGGAGGA-3′ and 5′-CCGTGTCAG CTCAGTTCTCAG-3′. The PCR products were subcloned into a Gateway entry vector, pENTR/D-TOPO (Invitrogen). The inserts in the vector were transferred into an RNAi vector for plant research, pANDA (Miki and Shimamoto 2004), by an LR clonase reaction. The RNAi constructs were then introduced into rice (*O. sativa* cv. Nipponbare or the *cry1b-1* mutant) through an *Agrobacterium*-mediated procedure (Toki 1997). *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986) was used in this transformation. The transformants obtained were cultivated for at least two generations to establish homozygous transgenic lines (*cry1a-R*, *cry2-R* and *cry1a-R cry1b-1*).

Western blotting analysis

Rice seedlings grown in the dark for 3 d were harvested using a night vision device. Aerial parts of the seedlings were ground using a mortar and pestle in protein elution buffer (100 mM Tris–HCl, pH 8.3, 5 mM EDTA, 0.2% 2-mercaptoethanol; Nagatani et al. 1993) containing 1× Complete Protease Inhibitor Cocktail (Roche). Proteins in the extracts were precipitated with 40% saturated ammonium sulfate. After centrifugation, proteins in the precipitate were resuspended in the same buffer. The protein concentration of the extracts was determined using Coomassie PLUS Protein Assay Reagent (Pierce). A 50 μg aliquot of protein was subjected to SDS–PAGE using gels containing 12% (w/v) acrylamide. Separated proteins in the gels were blotted onto PVDF membranes (Millipore) electrophoretically. The antibodies used and the procedures for immunochemical detection followed an earlier study (Hirose et al. 2006) using BCIP/NBT Color Development Substrate (Promega).

Growth measurement of seedlings grown under blue light

Rice seedlings grown under various fluence rates (0.1, 1, 10 or 30 μmol $m^{-2} s^{-1}$) of B light for 8 d at 28°C were picked up and scanned with a flatbed scanner to obtain images for computer analyses. The lengths of their second leaf sheaths were measured from the images using ImageJ software.

The effect of exogenous GA_1 on leaf elongation

A microdrop assay (Murakami 1972) was conducted to examine the effects of exogenous GA_1 on the seedlings. For our purposes, the assay was slightly modified in terms of conditions of light treatments. A 1 μl drop of ethanol containing 10 ng of GA_1 (OlChemIm) was put onto shoots of WT and *cry1a-R cry1b-1* seedlings that were grown under B light on agar medium in the presence of 0.3 μM PAC (Wako Pure Chemical Industries) for 3 d. On the fifth day after the application of GA_1, images of the seedlings were acquired with a flatbed scanner and the lengths of the second leaf sheaths were measured using ImageJ image processing software.

Supplementary data

Supplementary data are available at PCP online.

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