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Involvement of Rice Cryptochromes in De-etiolation Responses and Flowering

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In order to elucidate the function of cryptochromes (cry) in rice, we have characterized all rice *CRY* genes, including *OsCRY1a*, *OsCRY1b* and *OsCRY2*. Our studies revealed that *OsCRY1* genes were mainly expressed in the green plant tissue, while *OsCRY2* gene expression was high in the coleoptile, flower and callus. Light treatment affected neither the expression of any of the *OsCRY* genes nor the stability of their transcripts. However, it was found that Oscry2 protein was negatively regulated by blue light. Moreover, the level of Oscry2 protein also decreased upon red light treatment, and this red light-dependent degradation was shown to be mediated by phytochrome B. Overexpression of *OsCRY1* genes resulted in an increased responsiveness to blue light when measuring coleoptile growth inhibition. Moreover, growth of leaf sheaths and blades was also repressed more in *OsCRY1* overexpressers than in wild type (WT) under blue light conditions. These results suggest that Oscry1s are responsible for regulating blue light-mediated de-etiolation in rice. In addition, *OsCRY2* antisense transgenic rice flowered later than WT under both long-day and short-day conditions, indicating that Oscry2 is involved in the promotion of flowering time in rice.

Keywords: Blue light — Coleoptile — Cryptochrome — Flowering — Rice.

Abbreviations: JA, jasmonic acid; CCT, cryptochrome C-terminal; CO, CONSTANS; LD, long day; SD, short day; WT, wild type.

The nucleotide sequences reported in this paper have been submitted to DDBJ as follows: *OsCRY1a* (AB073546), *OsCRY1b* (AB073547) and *OsCRY2* (AB103094).

Introduction

Cryptochromes are blue/UV-A light photoreceptors that mediate various light responses in plants and animals (Cashmore et al. 1999). Plant cryptochrome genes have been identified and characterized in various plant species, such as *Arabidopsis* (Ahmad and Cashmore 1993, Hoffman et al. 1996, Lin et al. 1998), tomato (Ninu et al. 1999, Perrotta et al. 2000, Perrotta et al. 2001, Giliberto et al. 2005), rice (Matsumoto et al. 2003), pea (Platten et al. 2005a, Platten et al. 2005b), fern (*Adiantum capillus-veneris*; Kanegae and Wada 1998, Imaizumi et al. 2000), moss (*Physcomitrella patens*; Imaizumi et al. 2002) and algae (*Chlamydomonas reinhardtii*; Small et al. 1995).

Plant cryptochrome genes constitute a gene family and are categorized into two classes (*CRY1* and *CRY2*) in higher plants (Lin 2002). The amino acid sequences of CRY proteins revealed photolyase-like domains at the N-terminus, which are highly conserved among plant cryptochromes and have been shown to be required for homodimerization (Sang et al. 2005). The C-terminal extensions, named CCT (cryptochrome C-terminal) domains, demonstrate sequence diversity, and are thought to play important roles in signal transduction mechanisms (Guo et al. 1999, Yang et al. 2000, Wang et al. 2001). Functional analysis of cryptochromes (cry1 and cry2) has mostly been carried out in *Arabidopsis* by using *cry1* and *cry2* mutants (Ahmad and Cashmore 1993, Guo et al. 1998, Lin 2002), as well as transgenic plants (Ahmad et al. 1998, Lin et al. 1998, Yang et al. 2000). In *Arabidopsis*, cry1 is considered as the major blue light receptor regulating de-etiolation. Cry2 also mediates blue light-dependent regulation of the de-etiolation response, but its contribution is limited to a low light intensity range. In addition to its function in de-etiolation, cry2 also plays a role in the regulation of flowering time. The *Arabidopsis cry2* mutant has been shown to be allelic to the photoperiod-hyposensitive late-flowering mutant *fha* (Koornneef et al. 1991, Guo et al. 1998). Recently, a new cryptochrome class (CRY-DASH) has been identified in *Synechocystis* (Brudler et al. 2003), which was the first cryptochrome identified from bacteria. The CRY-DASH members have been identified in a wide range of organisms, including *Arabidopsis* (Brudler et al. 2003, Kleine et al. 2003), *Vibrio cholerae* (Worthington et al. 2003), *Neurospora crassa* (Daiyasu et al. 2004), zebrafish (Daiyasu et al. 2004) and *Xenopus laevis* (Daiyasu et al. 2004). Their biological functions remain unknown.

It has been known that blue light is essential for the proper growth of rice plants. A recent study (Matsuda et al. 2004) has shown that supplemental blue light increased the total nitrogen content of rice leaves, resulting in the

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enhancement of both light-saturated and light-limited photosynthesis. Recently, rice cryptochromes were isolated and their intracellular localization was characterized in cells from *Arabidopsis* (Matsumoto et al. 2003). However, the function of rice cryptochromes in planta is not well understood. To date, our group has isolated and characterized all phytochrome mutants from rice to investigate various photomorphogenic responses (Takano et al. 2001, Takano et al. 2005). In order to obtain a comprehensive understanding of the light-regulated responses in rice, it is vital to elucidate the signal transduction pathways mediated by blue light receptors.

In the present study, we isolated and characterized three cryptochrome genes (*OsCRY1a*, *OsCRY1b* and *OsCRY2*) from rice and found that expression of the *OsCRY1* and *OsCRY2* genes shows different tissue specificities. It was also revealed that Oscry2 protein is negatively regulated by blue light. We therefore produced transgenic rice, in which either the *OsCRY1a* or *OsCRY1b* gene was overexpressed, and analyzed the effect of blue light on early photomorphogenesis in rice. Following irradiation with a very low fluence of blue light, the *OsCRY1a* or *OsCRY1b* overexpressers demonstrated inhibition of coleoptile elongation, whereas there was a lack of inhibition in wild-type (WT) plants, indicating an increase in sensitivity to blue light in the transgenic rice. These results suggest that Oscry1a and Oscry1b are involved in blue light-mediated inhibition of coleoptile elongation in rice. In addition, *OsCRY2* antisense transgenic rice flowered later than the WT under both long-day (LD) and short-day (SD) conditions, suggesting that Oscry2 is involved in promoting flowering time in rice.

Results

Characterization of rice cryptochrome genes

We isolated two independent full-length cDNA clones from a rice cDNA library for *Arabidopsis Cryptochrome1* (*AtCRY1*) orthologs, which were named *OsCRY1a* (AB073546) and *OsCRY1b* (AB073547). Matsumoto et al. (2003) reported the characterization of rice CRY genes named *OsCRY1* (AB024337) and *OsCRY2* (AB098568), which correspond to *OsCRY1a* and *OsCRY1b*, respectively. However, discrepancies were found between the sequences for *OsCRY1* and *OsCRY2* reported by Matsumoto et al. (2003) and both the *OsCRY* sequences determined by our group, and rice genome sequences published by the Rice Genome Research Program (http://rgp.dna.affrc.go.jp/). Therefore, it can be assumed that the amino acid sequences for the *OsCRY1* and *OsCRY2* genes deduced by Matsumoto et al. (2003) are also incorrect. We also isolated the rice ortholog of *AtCRY2* cDNA, which was named *OsCRY2* (AB103094). Later, the full-length sequence for

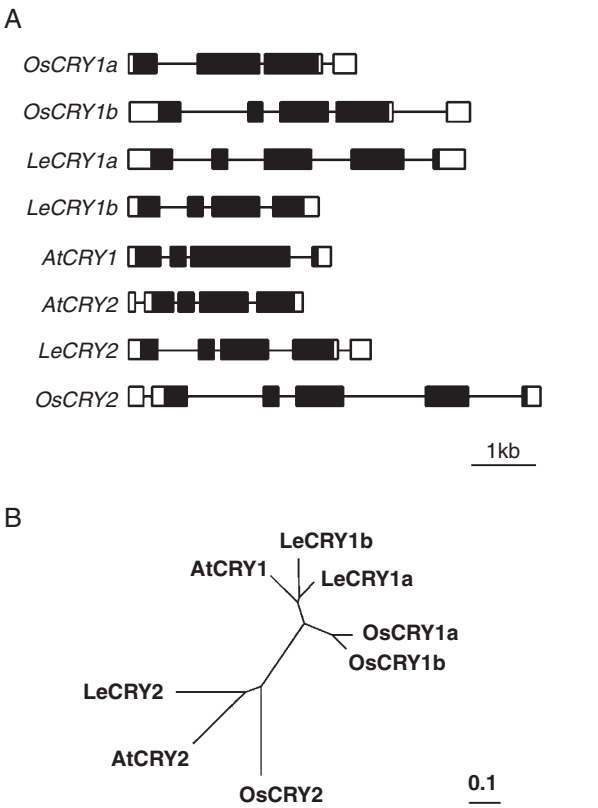

Fig. 1 Gene family of plant cryptochromes. (A) Gene structures of cryptochromes from higher plants. Boxes indicate exons and coding regions are filled in black. Corresponding exon borders are connected by thin lines. (B) Phylogenic tree showing the relationship among the higher plant cryptochromes. The relationship is based upon comparison of amino acid sequences using the CLUSTAL W program (http://www.ebi.ac.uk/clustalw/index.html). Amino acid sequences of AtCRY1 (S66907) and AtCRY2 (U43397) from *Arabidopsis,* and LeCRY1a (AF130423), LeCRY1b (AF348461) and LeCRY2 (AF130425) from tomato were obtained from the database. The branch lengths are proportional to the sequence divergence. The scale represents 0.1 substitutions per site.

OsCRY2 (AK065669) was obtained from the rice full-length cDNA database (KOME, http://cdna01.dna.affrc.go.jp/cDNA/), and it was revealed that 363 bp of 5′-untranslated sequence were truncated in our clone (AB103094).

The *OsCRY1a* gene consists of four exons, while *OsCRY1b* consists of five exons, the second of which is separated by an additional intron; the *OsCRY2* gene is composed of six exons (Fig. 1A). The complete sequences for *OsCRY1a*, *OsCRY1b* and *OsCRY2* cDNAs contain open reading frames of 710, 700 and 651 amino acids, respectively. OsCRY2 demonstrates greater homology with AtCRY2 (53% identity) than with either OsCRY1a or OsCRY1b (46 and 47% identity, respectively). The N-terminal, photolyase-like domains of all three proteins

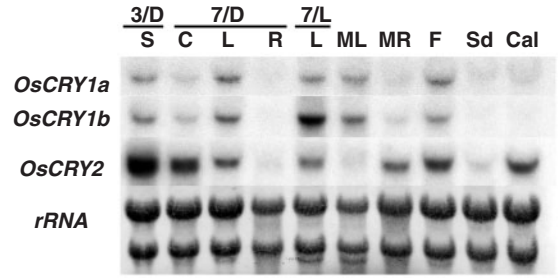

Fig. 2 Expression patterns of *OsCRY1a, OsCRY1b* and *OsCRY2* genes in various plant tissues. A 5 μg aliquot of total RNA isolated from a variety of plant tissues was electrophoresed and blotted to the membrane. RNA blots were hybridized with specific probes for the *OsCRY1a, OsCRY1b* or *OsCRY2* genes. Examined tissues were shoot (S) of 3-day-old dark-grown seedlings (3/D), coleoptiles (C), leaves (L) and roots (R) of 7-day-old dark-grown seedlings (7/D), leaves (L) of 7-day-old light-grown seedlings (7/L), leaves (L) and root (R) of mature plants (Mature), flower (F), immature seeds (Sd) and callus (Cal). Blotted membranes were stained with methylene blue for the detection of rRNA. All three membranes showed essentially the same staining pattern; a representative experiment is shown.

contain the regions of highest homology, with 88% identity between OsCRY1a and OsCRY1b, and 59% identity between OsCRY1a/OsCRY1b and OsCRY2. In contrast, the overall similarity among CCT domains is quite low (e.g. 16% identity between OsCRY1s and OsCRY2, Supplementary Table 1); however, several conserved motifs are still apparent (Supplementary Fig. 1). Of these, the DQXVP motif was firstly noted in *Adiantum* cryptochromes by Kanegae and Wada (1998), and is conserved across all OsCRY protein sequences. In addition, the STAESSS motif, which has been implicated in phytochrome A (phyA)-mediated phosphorylation (Ahmad et al. 1998), is not conserved in OsCRY proteins, but there are several S/T-rich motifs in the OsCRY sequences.

The chromosomal locations of *OsCRY1a*, *OsCRY1b* and *OsCRY2* genes were identified using the rice genome database (http://ricegaas.dna.affrc.go.jp/). *OsCRY1a* and *OsCRY2* genes reside on chromosome 2 (86.8 and 101.2 cM, respectively), while *OsCRY1b* is located on chromosome 4 (67.8 cM).

Expression of three rice cryptochrome genes

We determined the transcript levels of *OsCRY1a*, *OsCRY1b* and *OsCRY2* in various rice plant tissues. Total RNA was isolated from the shoots of 3-day-old dark-grown seedlings, from the coleoptiles and leaves of 7-day-old dark- or light-grown seedlings, and from mature leaves, roots, flowers, immature seeds and calli. As shown in Fig. 2, the expression of both *OsCRY1a* and *OsCRY1b* was greater in leaves than in any other tissue, while *OsCRY2* was strongly expressed in coleoptiles, flowers and calli. Interestingly, whereas *OsCRY2* was preferentially expressed in seedling leaves, in the mature plants expression was highest in root tissue. We do not yet know the significance of *OsCRY2* expression in roots. We then went on to examine the effects of light on the expression of *OsCRY1a*, *OsCRY1b* and *OsCRY2* genes. Etiolated 7-day-old seedlings were exposed to white light, and transcript levels of the *OsCRY* genes were monitored in the coleoptiles, leaves and roots during up to 4 h of light exposure. The results showed that the transcript levels for all *OsCRY* genes remained unchanged during light treatment in every plant tissue (data not shown). Therefore, expression of *OsCRY* genes is not regulated by light at least over a period of 4 h.

Light stability of OsCRY proteins

To enable further protein analysis to be carried out, we raised antibodies against the CCT domains of OsCRY1a, OsCRY1b and OsCRY2. To test the specificity of these antibodies, we generated Western blots using protein expressed in *Escherichia coli* (Supplementary Fig. 2A). Anti-OsCCT1a and anti-OsCCT2 antibodies were specific for OsCRY1a and OsCRY2 proteins, respectively. However, anti-OsCCT1b antibodies showed cross-reactivity with OsCRY1a. Fortunately, OsCRY1a and OsCRY1b have different molecular weights (predicted values: 79.2 and 78.8 kDa, respectively), which therefore makes it possible to distinguish between these two proteins. Results from Western blots of protein extracts from overexpressers and antisense plants showed that the expression of OsCRY1a and OsCRY1b was increased in overexpressers and decreased in extracts from antisense plants, providing further evidence that the antibodies generated are specific for CRY proteins (Supplementary Fig. 2B).

We also investigated the effect of light on the levels of Oscry1a, Oscry1b and Oscry2 proteins. Protein was extracted from 7-day-old dark-grown seedlings or from those exposed to 0.5, 1, 2, 4, 12 or 24 h white light. In 7-day-old dark-grown seedlings, all three cryptochrome proteins were present at detectable levels. When the seedlings were exposed to white light, Oscry2 protein was reduced rapidly, and became undetectable after 2 h exposure (Fig. 3A). In contrast, Oscry1a and Oscry1b were still detectable after 24 h irradiation (Fig. 3A), and also in the 7-day-old light-grown seedlings (data not shown).

According to Ahmad et al. (1998) and Lin et al. (1998), cry2 protein is only negatively regulated by blue light. We examined the effects of light quality blue, red or far-red light) on the expression of Oscry2 protein. As observed under white light irradiation, Oscry2 protein declined rapidly following blue light treatment (Fig. 3B). Far-red light had no effect on Oscry2 protein expression, but exposure to red light resulted in a gradual reduction over time (Fig. 3B). In *phyB* mutants, the red light-mediated

reduction in Oscry2 expression was not observed, suggesting that this effect was mediated by phyB.

Closer examination of Oscry1a protein levels revealed that exposure to white light for 0.5 h caused a rapid decrease, with constant levels of expression maintained thereafter (Fig. 3A). Blue light was also observed to cause a reduction in Oscry1a level, whereas red and far-red light had no effect (Fig. 3C). The blue light-mediated down-regulation of Oscry1a occurred extremely rapidly, with a reduction in protein expression to the basal level recorded in an exposure time of <10 min (Fig. 3D).

Possible involvement of rice cryptochromes in light-induced inhibition of coleoptile growth

The elongation growth of rice coleoptiles is inhibited by blue light, and phytochrome has been shown to be involved in this response (Pjon and Furuya 1967). In order to evaluate a possible contribution of cryptochrome to blue light-induced coleoptile growth inhibition, we constructed *OsCRY1* gene overexpressers (*OsCRY1a*OE and *OsCRY1b*OE) and compared the blue light-induced inhibition of coleoptile growth between these overexpressers and WT plants. We made approximately 20 independent transgenic lines for each construct and selected several lines expressing higher levels of either Oscry1a or Oscry1b protein (*OsCRY1a*OE#9 and #10, *OsCRY1b*OE#16 and #17) for further analysis. As compared with the WT, two of the selected *OsCRY1a* overexpresser lines, *OsCRY1a*OE#9 and #10, showed 50 and 25 times greater levels of Oscry1a protein, respectively; two of the selected *OsCRY1b* overexpresser lines, *OsCRY1b*OE#16 and #17, showed two and 50 times greater levels of Oscry1b protein, respectively (Supplementary Fig. 2C).

Using the overexpressing transgenic lines, we examined the effects of a brief irradiation with blue light (450 nm, 10^{-1}–10^{2} μmol m^{-2}) on the elongation growth of coleoptiles. Fluence–response curves were plotted for the WT, *OsCRY1a*OE and *OsCRY1b*OE, as shown in Fig. 4A. In the WT, 10 μmol m^{-2} blue light caused a partial inhibition of coleoptile growth (65% of control), and approximately 50% inhibition was obtained at 100 μmol m^{-2}. Conversely, lower fluences (0.1 and 1 μmol m^{-2}) were effective in the *OsCRY1* overexpressers; a 40–60% inhibition was obtained in these overexpressers following exposure to 1 μmol m^{-2} blue light. These results indicated that increased expression of Oscry1 resulted in enhanced responsiveness of coleoptile to blue light, and that Oscry1a and Oscry1b functioned similarly when overexpressed. It was noted that *OsCRY1b*OE#*16*, which showed the lowest level of Oscry1 protein overexpression, was less sensitive to blue light than the other higher expressing lines, indicating that the level of protein expression is correlated with the extent of the blue light response.

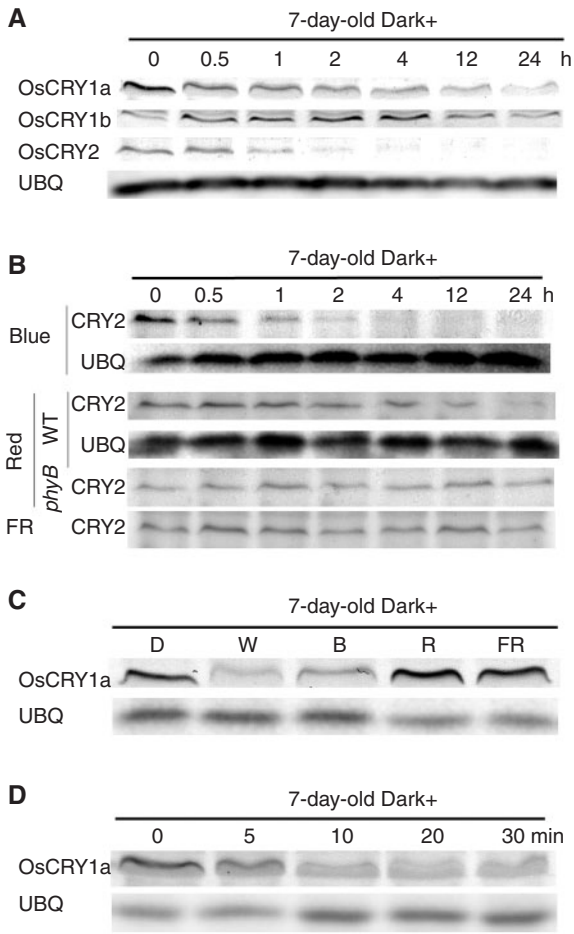

Fig. 3 Amounts of Oscry1a, Oscry1b and Oscry2 proteins after various light treatments. (A) Seven-day-old etiolated seedlings were exposed to white light (50 μmol m^{-2} s^{-1}) for the indicated periods before harvesting. A 50 μg aliquot of protein for each sample was fractionated and blotted. Oscry1a, Oscry1b or Oscry2 protein was detected using specific antibodies. Rice ubiquitin 1 proteins detected using the specific antibody were used as a control for loading. (B) Seven-day-old etiolated seedlings were exposed to blue light (1.8 μmol m^{-2} s^{-1}), red light (12 μmol m^{-2} s^{-1}) or far-red light (FR; 11 μmol m^{-2} s^{-1}) for the indicated periods before harvesting. In addition to Nipponbare (WT), rice *phyB-1* mutants were also grown and treated by red light for immunoblotting. A 50 μg aliquot of protein for each sample was fractionated and blotted, and Oscry2 proteins were detected using a specific antibody. (C) An immunoblot showing Oscry1a protein from 7-day-old etiolated seedling exposed to white light (W; 50 μmol m^{-2} s^{-1}), blue light (B; 1.8 μmol m^{-2} s^{-1}), red light (R; 12 μmol m^{-2} s^{-1}) or far-red light (FR; 11 μmol m^{-2} s^{-1}) for 30 min, or kept in the dark (D). (D) Seven-day-old seedlings were exposed to white light (50 μmol m^{-2} s^{-1}) for the indicated periods before harvesting. Immunochemical detection of Oscry1a protein was performed as in (A).

Riemann et al. (2003) found that exposure to red light results in an enhanced level of jasmonic acid (JA). Haga et al. (2004) found that the *allene oxide synthase 1* gene of rice (*OsAOS1*), which encodes a key enzyme for

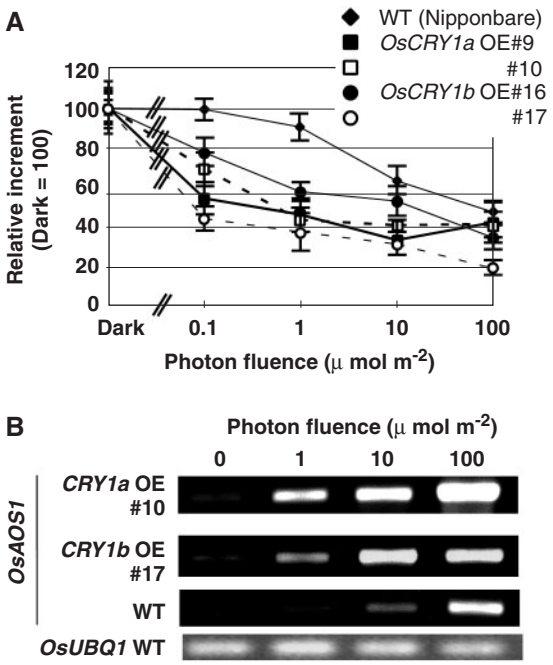

Fig. 4 Effects of blue light pulses with different photon fluences on the inhibition of coleoptile elongation and the expression of the *AOS1* gene. (A) Three-day-old etiolated Nipponbare seedlings (WT, filled diamonds with a thin line), *OsCRY1a*OE#9, #10 (filled squares with a thick line and open squares with a dashed thick line) and *OsCRY1b*OE#16, #17 (filled circles with a thin line and open circles with a dashed thin line) were exposed to blue light pulses with the indicated total fluences and then grown in the dark for 4 d. Coleoptile lengths were then measured. Error bars represent standard errors from 12–15 seedlings. (B) Three-day-old etiolated seedlings of *OsCRY1a*OE#10, *OsCRY1b*OE#17 and Nipponbare (WT) were exposed to a pulse of blue light (total fluences as indicated on the top: 0, 1, 10 or 100 μmol m^{-2}) and then kept in the dark for 1 h. Total RNA was extracted from coleoptiles and subjected to semi-quantitative RT–PCR analysis for the analysis of *OsAOS1* transcripts. The transcripts of the rice *Ubiquitin 1* gene (*OsUBQ1*) were amplified as internal controls. All three samples gave similar amplification patterns, and the results for the WT sample are shown.

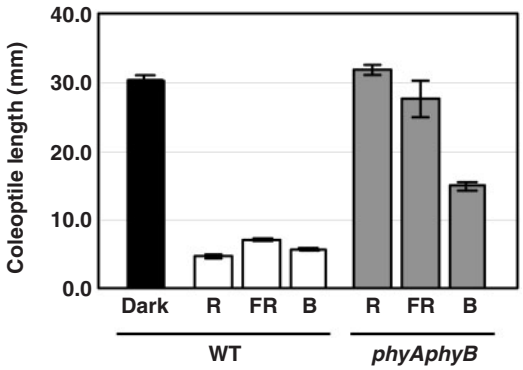

Fig. 5 Effects of continuous red (R), far-red (FR) and blue light (B) on the elongation growth of the coleoptiles of WT (Nipponbare) and *phyAphyB* double mutant seedlings. Seedlings were grown continuously at 28°C under the indicated light for 9 d after sowing on 0.4% (w/v) agar. The final length of coleoptiles achieved by the end of this growth period was determined. The fluence rates used were 15 μmol m^{-2} s^{-1} for R, FR and B. Control WT plants were grown in the dark. The means ± SE were obtained from 20–50 seedlings.

JA biosynthesis, is induced by red light. These lines of evidence have suggested that JA participates in phytochrome-mediated inhibition of rice coleoptile elongation. There is a possibility that JA is also involved in the blue light-induced coleoptile growth inhibition. To test this possibility, we examined the effect of blue light on the level of *OsAOS1* transcript in the WT and the *OsCRY1*-overexpressing coleoptiles. The transcript level increased with increasing fluences of blue light from 10 to 100 μmol m^{-2} in WT coleoptiles. In *OsCRY1a*OE and *OsCRY1b*OE, *OsAOS1* expression was induced clearly at a lower fluence (1 μmol m^{-2}) and the levels of expression at 10 and 100 μmol m^{-2} were generally greater than in the WT (Fig. 4B). These results are in agreement with those obtained for the blue light-induced inhibition of coleoptile growth (Fig. 4A). This agreement supports the idea that the induction of *OsAOS1*, and hence the resulting increase in JA biosynthesis, is involved in the Oscry1a- and Oscry1b-mediated inhibition of coleoptile growth, at least in the investigated overexpressers.

We previously found that the coleoptile of a *phyAphyB* double mutant that lacks both phyA and phyB also has a substantially reduced amount of phyC (Takano et al. 2005). Using this *phyAphyB* double mutant, we examined the effects of continuous irradiation with red, far-red and blue light on coleoptile elongation. WT seedlings responded to all the light treatments and showed substantially shorter coleoptiles. As shown in Fig. 5, the *phyAphyB* double mutants did not show any significant response to red and far-red light. However, blue light clearly inhibited coleoptile elongation in the double mutants, although the extent of inhibition was relatively smaller than that found in WT seedlings. These results demonstrate that the blue light-induced inhibition of coleoptile growth involves both phytochromes and blue light-specific photoreceptors. The results do not demonstrate that the blue light-specific photoreceptors are cryptochromes, but agree with the suggested involvement of Oscry1a and Oscry1b in the blue light-induced inhibition of coleoptile growth.

Seedling phenotypes of OsCRY overexpressers under blue light

We analyzed the role of cryptochromes in the photomorphogenesis of rice seedlings by comparing *OsCRY*OE and Nipponbare (WT) seedlings grown for 9 d under continuous irradiation with far-red, red

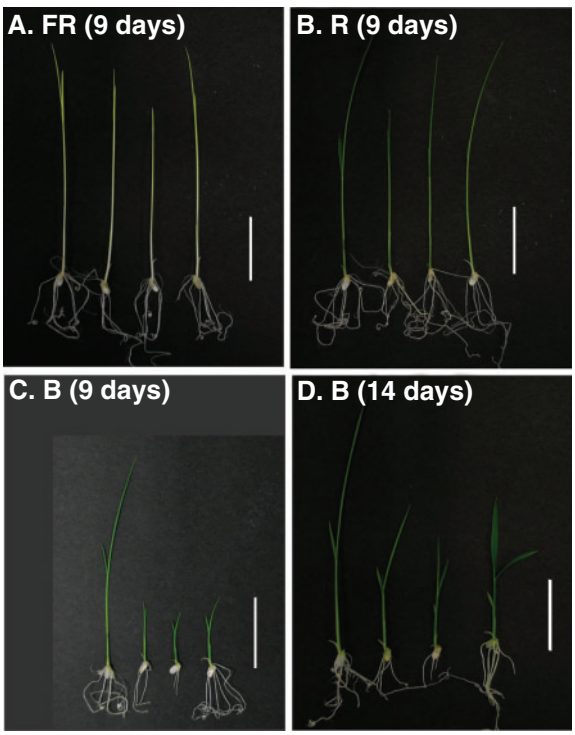

Fig. 6 Seedling phenotypes of WT and *OsCRY* overexpressers grown under far-red (A), red (B) or blue light (C) for 9 days, or under blue light for 14 d (D), at 28°C. The fluence rates of far-red, red and blue light are 15 µmol m^{-2} s^{-1}. WT and representatives of *OsCRY*OEs are aligned for comparison from left to right: WT, *OsCRY1a*OE#10, *OsCRY1b*OE#17 and *OsCRY2*OE#. All pictures are at the same magnification, and scale bars on the right are 10 mm.

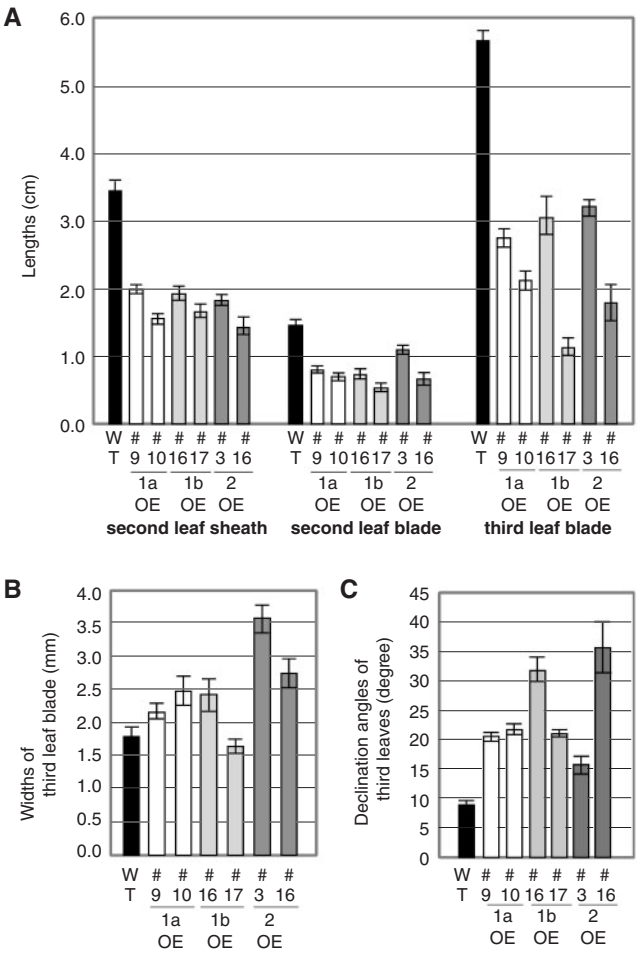

Fig. 7 Measurements of several parts of the 9-day-old or 14-day-old seedlings represented in Fig. 5. The lengths of leaf blades and sheaths (A), widths (B) and declination angles of the third leaf blades (C) of 14-day-old seedlings are shown for WT and *OsCRY*OE grown under blue light, as in Fig. 5. The means ± SE were obtained from 20–30 seedlings.

and blue light (Fig. 6). No apparent differences were observed between the WT and *OsCRY*OE seedlings under far-red and red light (Fig. 6A, B) as well as under the control dark condition (data not shown). Under blue light, WT seedlings showed shorter leaf sheaths and blades than those grown under far-red or red light; these phenotypes observed under blue light were much more pronounced in *OsCRY*OE (Fig. 6C).

To quantify the differences, we took comprehensive measurements of various parts of the seedlings of WT and *OsCRY*OE grown under blue light for 14 d (Fig. 7), because third leaf blades were not fully expanded in the 9-day-old *OsCRY*OE seedlings. The lengths of second leaf sheaths, and both second and third leaf blades of *OsCRY*OE were significantly shorter than those of WT seedlings, when grown under blue light (Fig. 7A). On the other hand, leaf blades in the *OsCRY*OE seedlings were wider than those of the WT (Fig. 7B). With the exception of *OsCRY1b*OE#17, all overexpressers had wider leaf blades than WT controls. In the *OsCRY1b*OE#17 line, the phenotype was so severe that emergence of the third leaf was retarded, resulting in small leaf blades (Fig. 7A, B). Another significant phenotypic difference was observed in the leaf blade angles of third leaves when grown under blue light (Figs. 6D, 7C). We took pictures of seedlings and measured the declination angles of third leaf blades; continuous exposure to blue light increased leaf blade declination, and the effects were significantly greater in the *OsCRY*OE seedlings (Fig. 7C).

These results indicate that Oscry proteins may function as blue light photoreceptors, mediating the inhibition of leaf sheath and blade elongation, the expansion of leaf blade width and the increase in leaf blade declination.

Flowering time of the OsCRY2 antisense transgenic rice

In *Arabidopsis*, *cry2* mutants show late flowering under LD conditions (Guo et al. 1998). We were curious to know how Oscry2 is involved in the initiation of flowering in SD plants such as rice. Fig. 7A shows the flowering times for

two independent lines of *OsCRY2* antisense transgenic rice (*OsCRY2*AS#6 and #9), under either LD or SD conditions. *OsCRY2*AS#6 had a reduced amount of cry2 protein via an antisense expression (Fig. 7B) and showed late flowering under both LD and SD conditions. These observations suggest that Oscry2 is involved in the promotion of flowering time in rice under both SD and LD conditions. Another line, OsCRY2AS#9, showed the same flowering time as the WT under SD conditions and flowered slightly later than WT under LD conditions, but this line contained an almost equivalent amount of cry2 protein to the WT (Fig. 7B).

Discussion

Structures of cryptochrome genes

To date, plant cryptochrome genes have been reported from *Arabidopsis* (Ahmad and Cashmore 1993, Hoffman et al. 1996, Guo et al. 1998) and tomato (Perrotta et al. 2000, Perrotta et al. 2001). Comparison of these genes with those of *OsCRY* (Supplementary Table 1 and Fig. 1A) revealed that the coding regions of the N-terminal, photolyase-related domains are conserved at both the sequence and gene structure levels. The photolyase-related domains are interrupted by three introns, and the positions of these introns are completely conserved in all except *OsCRY1a* and *AtCRY1*, which lack the second and third introns, respectively. The lack of these introns is probably the result of evolutionarily new losses. In contrast, the C-terminal extensions vary significantly in their gene structures (position of introns), as well as in terms of their nucleotide sequences.

Lin (2002) proposed that plant cryptochromes have three conserved motifs in the C-terminal domain, collectively designated the DAS motif (DQXVP-acidic residues-STAES). Among them, the DQXVP motif is completely conserved in *CRY* genes reported from angiosperms to mosses (Ahmad and Cashmore 1993, Kanegae and Wada 1998, Lin et al. 1998, Perrotta et al. 2000, Perrotta et al. 2001). We have also reported such conservation in rice *CRY* genes, suggesting a fundamental role for this motif in cryptochrome function (Lin 2002). Ahmad et al. (1995) have reported that a mutation in the DQXVP motif (P_{549}–L) resulted in almost complete elimination of cry1 activity in the *hy4-9* mutant allele in *Arabidopsis*. The putative protein phosphorylation site, the STAES motif, is not conserved in rice as it is found in *Arabidopsis*; however, similar sequences are found in the corresponding positions: STSEASS (OsCRY1a), SVSEASS (OsCRY1b) and SSKMEATSS or SYSSSAE (OsCRY2). However, the acidic E/D motif is not clearly recognized in any of the rice *CRY* genes.

Expression patterns of rice cryptochrome genes

The expression patterns for cryptochrome genes (Fig. 2) showed that *OsCRY1a* and *OsCRY1b* were mainly expressed in the green tissues (leaf and flower), while *OsCRY2* was highly expressed in the coleoptiles, and significant expression was also observed in the flowers and calli. The expression pattern for *OsCRY2* is similar to that of the rice *PHYA* gene, which is also highly expressed in the coleoptiles. In addition, the product of *PHYA* is light labile. In *Arabidopsis*, cry2 mediates blue light-dependent early photomorphogenesis under low light intensity. Therefore, it seems plausible that Oscry2 is involved in mediating the coleoptile inhibition seen under low intensities of blue light, in the same way that phyA mediates the effects of red or far-red light (Takano et al. 2001).

Light stability of CRY proteins in rice

The Oscry2 protein level was reduced rapidly under white or blue light, and became undetectable after 2 h of irradiation (Fig. 3A). However, *OsCRY2* transcript levels were not affected by blue light irradiation (data not shown), suggesting that the reduction in protein resulted from a post-transcriptional event, possibly degradation, as demonstrated in *Arabidopsis* by Ahmad et al. (1998). Unlike the *Arabidopsis* cry2, Oscry2 protein expression also decreased upon red light irradiation, although the reduction was slower compared with blue light (Fig. 3B). The effect of red light was not observed in *phyB* mutants, indicating that phyB mediated the reduction in Oscry2 protein. No difference was observed between the *phyB* mutant and WT for the blue light-induced decline in Oscry2 protein (data not shown). These results suggest that blue and red light mediate the down-regulation in Oscry2 protein expression by separate pathways.

We also found that a part of the Oscry1a protein decreased very rapidly following exposure to blue light (Fig. 3C and D). This observation suggests the existence of two types of Oscry1a proteins, one being light labile and the other being light stable. Alternatively, the difference in the light stability of Oscry1a could be tissue dependent, since the protein was extracted from different tissues, including leaf blades and sheaths. At present, we are not able to determine the physiological significance of this blue light-dependent rapid decrease on Oscry1a protein.

Roles played by cryptochromes in seedling photomorphogenesis

In *Arabidopsis*, blue light-induced inhibition of hypocotyl elongation has been shown to be mediated by multiple photoreceptors, phyA and cry1 being the major photoreceptors involved (Poppe et al. 1998). Pjon and Furuya (1967) reported earlier that blue light-induced inhibition of rice coleoptiles can be reversed by far-red light,

demonstrating that phytochrome is a photoreceptor of this response. However, a part of the response could not be reversed by far-red light. It has been demonstrated that a far-red light-inducible very low fluence response participates in the red light-induced inhibition of rice coleoptile growth (Takano et al. 2001, Biswass et al. 2003). This type of response, shown to be mediated solely by phyA, is likely to be induced by blue light in a far-red light-irreversible manner. Another possibility is that other blue light-specific photoreceptor(s) participate in the blue light-induced inhibition of coleoptile growth. In fact, our results obtained with the *phyAphyB* double mutant demonstrate that blue light-specific photoreceptor(s) are indeed involved in the growth inhibition (Fig. 5). We found that transgenic rice overexpressing either *OsCRY1a* or *OsCRY1b* show greater response to blue light, suggesting that these cryptochromes are involved in the blue light-induced inhibition of coleoptile growth. Future study should examine whether OsCRY1a and OsCRY1b are the sole photoreceptors responsible for the blue light-specific response. It also remains to be examined whether phytochromes and cryptochromes play a greater role in the blue light-induced response.

Overexpression of *OsCRY1a*, *OsCRY1b* or *OsCRY2* resulted in reduced elongation of leaf sheath and blade (Fig. 7A). On the other hand, the leaf blade width appeared to be enhanced in these overexpressers (Fig. 7B). Furthermore, the declination angle of the third leaf blade was greater in the overexpressers (Fig. 7C). These results suggest that rice cryptochromes function as blue light-specific photoreceptors for the inhibition of leaf elongation, expansion of the leaf blade and declination of the leaf blade at least in the seedling stage. We could not observe any fundamental difference between the transgenic plants overexpressing *OsCRY1* and *OsCRY2* in these investigated aspects. This result, however, does not necessarily mean that Oscry1 and Oscry2 have the same functional roles.

Our fuller understanding of the role played by rice cryptochromes in seedling photomorphogenesis will depend on the availability of mutants of all *OsCRY* members and of double and triple mutants generated from these mutants.

Roles played by Oscry2 on the control of flowering time

Our data demonstrate that *OsCRY2* antisense transgenic rice flowered later than the WT under both SD and LD conditions (Fig. 8). Given that Oscry2 and phyB function antagonistically in regulating flowering time, as seen in *Arabidopsis* (Guo et al. 1998), then a late flowering phenotype for *OsCRY2* antisense plants would be expected, because rice *phyB* mutants show early flowering under both SD and LD conditions (Takano et al. 2005).

In *Arabidopsis*, phyB mediates the red light-dependent inhibition of flowering, and cry2 mediates the blue light-dependent inhibition of the phyB effect (Guo et al. 1998). The antagonistic functions of cry2 and phyB in the regulation of CO (CONSTANS) stability (Valverde et al. 2004) have been used as the basis to explain these observations, with the inference that the accumulated CO induces *FT* (*FLOWERING LOCUS T*) expression under LD conditions, and hence promotes flowering. In contrast, *Hd1* (rice ortholog of *CO*) was proposed to have two independent and opposite functions in the control of flowering time through the regulation of the *Hd3a* gene (rice ortholog of *FT*), promoting *Hd3a* expression under SD but inhibiting it under LD conditions (Yano et al. 2000, Izawa et al. 2002, Hayama et al. 2003). Moreover, additional *trans*-acting factors have been assumed in rice, which are responsible for a general up-regulation of *Hd3a* expression independently of Hd1 activity (Doi et al. 2004). Therefore, our results indicate that Oscry2 is involved in the promotion of flowering time under both LD and SD conditions, but the molecular mechanism underlying this effect may be more complicated in rice than in *Arabidopsis*.

We have been searching for *Oscry2* mutants from 'Mutant Panels' (Hirochika, 1999) with no success so far. Detailed analysis of *Oscry2* mutants grown under various

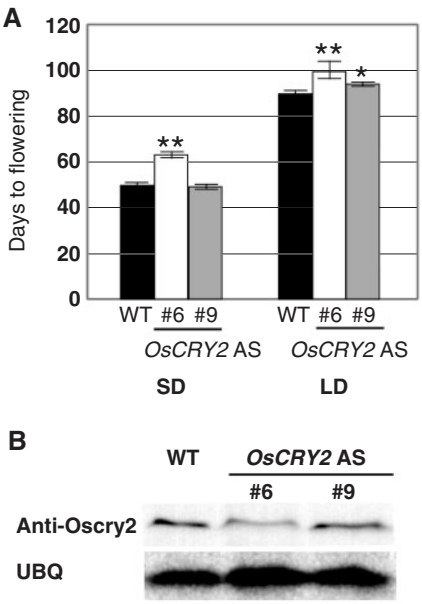

Fig. 8 Effect of LD and SD photoperiods on flowering time in *OsCRY2*-repressed transgenic plants. (A) The LD (long-day) condition is a light regime of 14.5 h light/9.5 h dark, and the SD (short-day) condition is 10 h light/14 h dark. The means ± SE were obtained from 6–15 plants. (*) and (**) indicate significant statistical differences by *t*-test ($P<0.05$ and $P<0.01$, respectively) between WT and *OsCRY2* antisense lines. (B) The amounts of Oscry2 protein were estimated by Western blotting for WT and *OsCRY2* antisense lines #6 and #9. Rice ubiquitin protein was used as an internal control.

light conditions, including different light quality as well as photoperiods, would provide important information to help understand the function of Oscry2 in the photoperiodic flowering responses of rice.

Concluding remarks

The blue light-absorbing photoreceptors cry1 and cry2 were uncovered in *Arabidopsis* and their functions have been investigated most extensively in this plant species (Lin 2002). Our results indicate that rice has two homologs of cry1 (Oscry1a and Oscry1b) and a homolog of cry2 (Oscry2). In *Arabidopsis*, cry1 is involved in blue light-induced inhibition of hypocotyl growth; cry2 is also involved but only at low fluence rates (Lin et al. 1998). Rice transgenic plants overexpressing *OsCRY1a* or *OsCRY1b* showed greater responsiveness to a pulse of blue light, and those overexpressing *OsCRY1a*, *OsCRY1b* or *OsCRY2* showed reduced leaf elongation under continuous blue light. Our results do not resolve which cryptochrome species is more functional, but suggest that Oscry1a and Oscry1b, and possibly also Oscry2, mediate at least a part of the blue light-dependent inhibition of coleoptile and leaf elongation. In *Arabidopsis*, the *cry2* mutation results in delayed flowering under a flowering-inducing LD condition. We found that *OsCRY2*-repressed transgenic plants show delayed flowering under a flowering-inducing SD condition. These results suggest that cry2 and its homologs are common higher plant photoreceptors involved in the photoperiodic control of flowering. We found that the level of Oscry2 is post-transcriptionally down-regulated by light as found for *Arabidopsis* (Ahmad et al. 1998, Lin et al. 1998). In Arabidopsis, this photoregulation is blue light specific. In contrast, the corresponding regulation in rice involves both the blue light-specific rapid response and the red light-inducible phyB-mediated slow response.

Materials and Methods

Isolation and sequence analysis of cDNA clones

A cDNA library was constructed using a lambda ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA, USA), using mRNA from 6-day-old light-grown rice (*Oryza sativa* L. cv. Nipponbare). The expressed sequence tag (EST) clone which showed homology with the *Arabidopsis cry1* gene (accession No: D41779) was used as a probe and, after screening, the two longest clones found with different restriction maps were chosen for determination of the complete sequences.

The CLUSTAL W program (Thompson et al. 1994) was used for alignment and comparison of CRY homologs, including OsCRY1a, OsCRY1b and OsCRY2.

Monochromatic light sources

Unless otherwise indicated, we used a red light-emitting diode panel (Model LED-R, EYELA, Tokyo, Japan), a far-red light-emitting diode panel (Model LED-FR, EYELA) and a blue light-emitting diode panel (Model LED-B, EYELA) for monochromatic light sources. The far-red light-emitting diode panel was fitted into a filter box with one layer of acryl cut-off filter (KYOWALITE PG; SP-60-3K 202; thickness = 2 mm; Kyowa Gas Chemical, Tokyo, Japan). White light was supplied by white fluorescent tubes (FL40SN-SDL, NEC, Tokyo, Japan).

RNA blot analysis

For mRNA detection from seedlings, dehusked seeds of Nipponbare were surface-sterilized and grown at 28°C for 3 or 7 d in complete darkness, or 7 d under continuous white light ($50\,\mu mol\,m^{-2}\,s^{-1}$). Total RNA was isolated from shoot parts (for 3 d in the dark), coleoptiles, leaves and roots (for 7 d in dark) or leaves (for 7 d in the light), using the RNeasy Plant Mini Kit (QIAGEN, GmbH, Germany). Total RNA was also isolated from leaves and roots of mature plants (2 months old), flowers, immature seeds and embryo-derived calli incubated for 2 weeks, by using the RNeasy Plant Mini Kit. For each tissue type, 10 μg of total RNA was electrophoresed on formaldehyde-containing gels and transferred to Hybond N^+ (Amersham Bioscience, Piscataway, NJ, USA). Specific probes were prepared from corresponding cDNAs by PCR amplification (5′-GAAACTCCATCAAATTCCCCA-3′ and 5′-GGACACTCCCAAATCAAGCAA-3′ for *OsCRY1a*, M13RV and 5′-CTTCCTTTCTTTTCCCGTGTA for *OsCRY1b*, and 5′-CCTTCACATTGCTAGGGAGT-3′ and 5′-GAAACCGT GTCAGCTCAGTT-3′ for *OsCRY2*).

Preparation of antibodies

In order to raise CRY-specific antibodies, we expressed the 3′-haf moieties of *CRY* cDNA (from 1,669 to 2,289 for *OsCRY1a*, from 2,004 to 2,609 for *OsCRY1b*, and from 1,540 to 2,184 for *OsCRY2*) in the *E. coli* protein expression system (pET16b, Novagen, Madison, WI, USA) as a histidine-tagged protein, and purified the protein by BD TALON Metal Affinity Resins (BD Biosciences, CA, USA). Rabbits were immunized with the C-terminal halves of CRY proteins (536–710 of the amino acid sequence of OsCRY1a; 535–710 of OsCRY1b; and 489–651 of OsCRY2). Full-length clones of *OsCRY1a* (AB073546), *OsCRY1b* (AB073547) and *OsCRY2* (AB103094) were also expressed in the *E. coli* system by using the pET16b vector. The expressed proteins were recovered as inclusion bodies and were partially purified by repeated sonication–centrifugation cycles. The specificity of the antibodies was confirmed by Western analysis, using expressed proteins in *E. coli* (Supplementary Fig. 2A) or crude protein extracts from 7-day-old etiolated seedlings of Nipponbare or transgenic plants (Supplementary Fig. 2B).

Western blot analysis

For the analysis of light effects on cryptochrome protein stability, rice seedlings were grown in the dark for 7 d and were exposed to continuous white ($50\,\mu mol\,m^{-2}\,s^{-1}$), red ($12\,\mu mol\ m^{-2}\,s^{-1}$), far-red ($11\,\mu mol\,m^{-2}\,s^{-1}$) or blue light ($1.8\,\mu mol\,m^{-2}\,s^{-1}$) for the indicated number of hours, and then harvested. Protein was extracted from whole seedlings by using protein extraction buffer (Nagatani et al. 1993) and precipitated with 60% saturated ammonium sulfate. The precipitated material was resuspended in protein extraction buffer, and protein concentrations were determined using Coomassie PLUS Protein Assay Reagent (Pierce, Rockford, IL, USA). All procedures described above were performed under an infrared LED light (SLR-938CV-7, Tottori SANYO Electric Co., Ltd., Tottori, Japan)

by means of Night vision. A 50 μg aliquot of protein was size fractionated by SDS–PAGE in 12% gels and then blotted onto PVDF membranes (Millipore, Billerica, MA, USA). Immunochemical analysis was performed as described in Takano et al. (2001).

Transgenic plants

Rice *CRY* full-length cDNAs were subcloned into the pIG121-Hm vector (Ohta et al. 1990), in which a transgene was driven by the *CaMV 35S* promoter, with sense (for *OsCRY1a, OsCRY1b* and *OsCRY2*) or antisense (for *OsCRY2*) orientation, and were then introduced into *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986) by electroporation. Rice (*O. sativa* L. var. Nipponbare) was transformed using the agro-infection methods developed by Toki (1997).

Blue light-induced suppression of coleoptile growth

In order to examine the effect of blue light on coleoptile growth, 3-day-old dark-grown seedlings of WT or *OsCRY1* overexpressers were exposed to a pulse of blue light with different fluences (0.1, 1, 10 or 100 $\mu mol\,m^{-2}$) and kept in the dark for 7 d. Images of individual seedlings just prior to blue light irradiation were taken by CCD camera in the dark, irradiating with infrared (950 nm) LED light, and were used for measuring the initial coleoptile lengths. After 7 d, the final coleoptile lengths were measured and the increments in length during the dark incubation were calculated for individual seedlings. Blue light was provided by a fluorescent lamp (FL20SB, Toshiba, Tokyo, Japan) filtered with an acryl color filter (Acrylight K5-102, Mitsubishi Rayon, Tokyo, Japan).

For quantifying *OsAOS1* transcripts, total RNA was extracted from the seedlings treated as above using the RNeasy Plant Mini Kit (QIAGEN, GmbH, Germany). 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, and one-twentieth of the reaction products was used directly for PCR amplification. The coding region of the *OsAOS1* gene was amplified by reverse transcription–PCR (RT–PCR) using gene-specific primers (AOS1/F5, 5′-GGTGTAGTTCATCGGATCAAGGGG-3′; AOS1/R3, 5′-GGGCGCCGGAGACTTTGACC-3′). After 30 cycles, the PCR products were examined by gel electrophoresis. Transcripts of the *OsUBQ1* gene were used as an internal control.

Measurements of plant parts

For the measurements of several parts of seedlings, sterilized seeds were sown onto 0.4% (w/v) agar and then grown in darkness or under continuous far-red (15 $\mu mol\,m^{-2}\,s^{-1}$), red (15 $\mu mol\,m^{-2}\,s^{-1}$) or blue light (15 $\mu mol\,m^{-2}\,s^{-1}$) at 28°C. The seedlings were removed after 9 d or 2 weeks, and their images were taken (see Fig. 5). Lengths and angles of seedling parts were measured from the images.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

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