Distinct and Cooperative Functions of Phytochromes A, B, and C in the Control of Deetiolation and Flowering in Rice

Makoto Takano, Noritoshi Inagaki, Xianzhi Xie, Natsu Yuzurihara, Fukiko Hihara, Toru Ishizuka, Masahiro Yano, Minoru Nishimura, Akio Miyao, Hirohiko Hirochika, and Tomoko Shinomura

INTRODUCTION

Plants perceive diverse light signals from the environment by a family of plant photoreceptors that includes phytochromes, cryptochrome, phototropins, and several others. The phytochromes are chromoproteins that regulate the expression of a large number of light-responsive genes and thus influence many photomorphogenic events (Neff et al., 2000; Quail, 2002a, 2002b; Wang and Deng, 2003).

Phytochromes in higher plants are encoded by small gene families (Clack et al., 1994; Mathews and Sharrock, 1997). Molecular phylogenetic analyses indicate that the angiosperm phytochrome gene family is composed of four subfamilies known as PHYTOCHROME A (PHYA), PHYB, PHYC/F, and PHYE (Alba et al., 2000). In Arabidopsis thaliana, PHYD was further derived from an ancestral PHYB gene by a recent gene duplication event (Clack et al., 1994), and as a result, Arabidopsis contains five PHY genes, PHYA to PHYE (Sharrock and Quail, 1989; Clack et al., 1994). The tomato (Lycopersicon esculentum) genome also has five PHY genes, PHYA, PHYB1, PHYB2, PHYE, and PHYF (Alba et al., 2000). On the other hand, monocotyledonous plants lack multiple members of PHYB subfamily, and rice (Oryza sativa) has only three genes, PHYA, PHYB, and PHYC (Kay et al., 1989; Dehesh et al., 1991; Tahir et al., 1998; Basu et al., 2000).

Phytochrome mutants have been crucial in determining the distinct roles of different members of the Arabidopsis phytochrome family (Quail, 1998; Whitelam et al., 1998). The first reported phytochrome mutant was that of phyB, which had been identified as a hy3 mutant (Koornneef et al., 1980) and displayed loss of the inhibition of hypocotyl elongation when grown in continuous red light (Somers et al., 1991). Mutants deficient in phyB have reduced sensitivity to red light and show constitutive shade-avoidance phenotype (Reed et al., 1993). Subsequently, phyA mutants were isolated by screening the long hypocotyl phenotype under far-red (FR) light (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). Analyses using phyA mutants (Shinomura et al., 1996) indicated that phyA is the only phytochrome that mediates seedling responses to continuous FR light and is also responsible for the very low fluence response (VLFR). It was not long before the ecotype Wassilewskija-2 was found to be deficient in functional phyD (Aukerman et al., 1997). Phylogenetic analysis revealed that PHYB and PHYD genes are closely related (Clack et al., 1994), and the phenotypes of the phyD mutant were more pronounced in a phyB null background. These lines of evidence suggested that phyD was involved in mediating the same responses as phyB and that there was some functional redundancy between these two phytochromes. Regarding the phyE mutant, because the monogenic mutation was not expected to cause distinguishable phenotypical changes, phyA phyB doubly null mutants were mutagenized by γ-rays, and M2 seedlings were screened for individuals displaying shade-avoidance response (Devlin et al., 1998). The phyA phyB

We have isolated phytochrome B (phyB) and phyC mutants from rice (Oryza sativa) and have produced all combinations of double mutants. Seedlings of phyB and phyB phyC mutants exhibited a partial loss of sensitivity to continuous red light (Rc) but still showed significant deetiolation responses. The responses to Rc were completely canceled in phyA phyB double mutants. These results indicate that phyA and phyB act in a highly redundant manner to control deetiolation under Rc. Under continuous far-red light (FRc), phyA mutants showed partially impaired deetiolation, and phyA phyC double mutants showed no significant residual phytochrome responses, indicating that not only phyA but also phyC is involved in the photoperception of FRc in rice. Interestingly, the phyB phyC double mutant displayed clear R/FR reversibility in the pulse irradiation experiments, indicating that both phyA and phyB can mediate the low-fluence response for gene expression. Rice is a short-day plant, and we found that mutation in either phyB or phyC caused moderate early flowering under the long-day photoperiod, while monogenic phyA mutation had little effect on the flowering time. The phyA mutation, however, in combination with phyB or phyC mutation caused dramatic early flowering.
photoperception, VLFR and high irradiance response, which are the functions in the regulation of the shade-avoidance syndrome. Forward genetics screening had not been successful for the identification of the phyC mutant. Recently, two groups have reported the isolation of phyC mutants from Arabidopsis by means of a reverse genetics approach (Franklin et al., 2003; Monte et al., 2003).

The diverse functions of phytochromes in the regulation of plant development have been characterized mainly in dicots using the mutants as mentioned above. However, little information in this regard is available in monocots, mainly because of the unavailability of phytochrome mutants. The generation and characterization of phytochrome mutants of rice can greatly enhance the existing knowledge of phytochrome function in plants, especially in discriminating phytochrome functionality between monocots and dicots. Therefore, we adapted a reverse genetics strategy to identify rice phytochrome mutants because the mutant phenotypes in rice have until this time remained unknown. The Rice Genome Research Program (http://rgp.dna.affrc.go.jp/) has generated a large population of retrotransposon (Tos17) insertion mutants in rice, and DNAs isolated from the mutant plants were organized as superpools and correspondingly designated as ”mutant panels” (Hirochika, 1997, 1999). The mutant panels were efficiently screened by PCR using PHYA and Tos17-specific primers, leading to the isolation of several alleles of phyA mutants (Takano et al., 2001, 2005).

Physiological analyses of the phyA mutants revealed that rice phyA controls photomorphogenesis in two distinct modes of photoperception, VLFR and high irradiance response, which are consistent with the results obtained for phyA mutants in Arabidopsis and tomato (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993; Reed et al., 1994; van Tuinen et al., 1995a). For example, phyA seems to play a similar role in the deetiolation of monocot and dicot plants, and FR light induces the inhibition of coleoptile elongation in rice and hypocotyl elongation in Arabidopsis. However, the modes of photoperception by phyA in those responses are different, the former being via VLFR and the latter being mainly via high irradiance response (Shinomura et al., 2000). Thus, comparing the phyA mutants of Arabidopsis and rice provided information about features that are common between the species or specific to each species.

We sequenced the phyB and phyC genes as well and isolated one mutant line of phyC after an extensive screening of the mutant panels (>50,000 mutant lines). For isolating phyB mutants, phenotypic screening has been performed for the M2 generation of a rice population mutagenized by γ-ray irradiation since no mutant line could be obtained from the mutant panels. We isolated five alleles of phyB mutants using this forward genetics strategy. In this article, we report the characterization of every single and double phytochrome mutant in terms of the deetiolation and flowering process in rice. In Arabidopsis, it is suggested that phyC is involved in photomorphogenesis, with a photosensory specificity similar to that of phyB/D/E (Monte et al., 2003). In rice, however, we found that phyC is involved in the photoperception of FR for the deetiolation as well as the induction of Lhcb (for light-harvesting chlorophyll a/b binding protein) genes and has little effect on the red light-mediated responses. Furthermore, we demonstrated that rice phyA is in charge of low fluence response (LFR) as well as VLFR. Finally, synergistic, redundant, or antagonistic effects of phytochromes were observed on the flowering-time determination in response to the daylength, and these effects differed from those in Arabidopsis.

RESULTS

Isolation of phyB and phyC Mutant Lines

We screened ~3000 M2 seedlings from γ-ray-mutagenized Nipponbare seedlings for an elongated coleoptile (elc) phenotype under continuous red light (Rc) irradiation in order to isolate rice phyB mutants. The screening yielded one elc mutant (elc-1) whose coleoptile was relatively straight and long compared with control seedlings and to almost all other M2 seedlings, which displayed curved and shortened coleoptiles. Rice phytochromes apparently function as a repressor of flowering induction under long-day (LD) conditions (Iizawa et al., 2000). Thus, we screened 37 lines of early flowering mutants, which had been isolated at the Institute of Radiation Breeding at the National Institute of Agrobiological Sciences, for the elc phenotype. We were able to obtain four elc alleles (elc-2, elc-3, elc-4, and elc-5) in this screening, and the averaged coleoptile lengths of elc mutants were clearly longer than those of wild-type seedlings under Rc irradiation. Background cultivar of the elc-2 is Nipponbare and that of elc-3, elc-4, and elc-5 is Norin 8.

We sequenced the PHYB genes from the elc mutants based on the idea that elc mutations were probably within the PHYB gene. As expected, we detected either a deletion or an insertion in the coding region of the PHYB gene from all elc mutants isolated in this work (Figure 1A). The insertion or deletions induced frame shifts and subsequently created new stop codons that caused immature translations (see Supplemental Figure 1 online), as revealed in the sequences of elc-1, elc-2, elc-4, and elc-5 mutants. These mutations are gross enough to abolish the normal function of phyB. However, the mutation of the elc-3 appeared to be less drastic because a deletion of 33 bp removed the stop codon along with a sequence corresponding to C-terminal 9–amino acid residues and added 62 irrelevant residues at the C terminus of PHYB. It is evident from these results that all the elc mutants reported in this work are phyB mutants; therefore, elc-1 to elc-5 were renamed as phyB-1 to phyB-5, respectively.

We estimated protein levels of phytochromes in the phyB mutants by protein gel blot analyses using antibodies specific to PHYA, PHYB, or PHYC proteins. Figure 1B shows that PHYB protein with expected molecular mass could not be detected in phyB-1, phyB-2, phyB-4, and phyB-5 mutants, while it was barely detectable in the phyB-3 mutant. Moreover, any additional bands in phyB-1, phyB-2, phyB-4, and phyB-5 mutants could not be detected, suggesting that these mutants in fact are null alleles. The band detected for the phyB-3 was shifted slightly upward, which seemed to be a consequence of the mutation that added extra 52–amino acid residues to the C terminus.

We isolated one rice phyC line by screening DNA pools of rice Tos17 insertion lines (mutant panels) using a PCR-based
screening strategy. The mutant line was found to have a Tos17 insertion in the first exon of the PHYC gene (Figure 1A). The insertion site corresponds to the position of the 244th amino acid, which is 77 amino residues upstream from the chromophore binding site (Cys). The Tos17 insertion was also confirmed by DNA gel blot hybridization. Neither PHYC transcript (data not shown) nor PHYC protein was detected in the phyC mutant (Figure 1D), indicating that it was a null mutation. The mutation for phyC was observed to be inherited and segregated as a single mutation in the F2 generation. We designated this mutant line as phyC-1.

We have already isolated phyA mutants from rice (Takano et al., 2001). To analyze the individual as well as interactive functions of phytochromes, we produced all combinations of double mutants having the same background (Nipponbare) by crossing phyA-2 or -4, phyB-1, and phyC-1 mutants (Figure 1D) and examined phenotypes and gene expression patterns of wild-type and the single and double mutants under various light conditions.

**PHYC Protein Levels in the phyB Mutants**

In Arabidopsis, several reports (Hirschfeld et al., 1998; Monte et al., 2003) showed that phyC levels were reduced in phyB seedlings. As shown in Figure 1B, we observed the reductions of PHYC protein levels in the rice phyB mutants as well, while protein levels of the PHYA in the mutants were almost comparable with those in the wild-type plants. The amount of PHYC proteins in phyB-1 was estimated to be less than one-eighth of those observed for the wild-type plants (Figure 1C). We also examined mRNA levels of PHYB and PHYC genes in phyB mutants (Figure 1B). Only faint bands were detected in phyB-1, phyB-2, phyB-4, and phyB-5 seedlings, whereas phyB-3 had levels of PHYB transcript similar to those of wild-type seedlings. Transcript levels of PHYC genes were not reduced in phyB mutants (Figure 1B), indicating that the reduction of PHYC protein is a posttranscriptional event.

These results mean that the phyB mutant and the phyA phyB double mutant lack the vast majority of their phyC (Figure 1E), and the phenotypes of these mutants should be interpreted with caution at this point. For example, the phenotypic changes seen in phyB mutants may reflect loss of activity of both phyB and phyC, whereas phenotypic changes seen in the phyA phyB double mutant likely reflect loss of activity of all three phytochromes.

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Figure 1. Characterization of phyB and phyC Mutants.

(A) Mutation sites found in the PHYB gene and insertion site of Tos17 in the PHYC gene are schematically depicted. Exons are represented as boxes in which closed parts are coding regions and open parts are 5’- and 3’-untranslated regions. The chromophore binding site is indicated by “#.” The horizontal arrow shows an orientation of the inserted Tos17 (gray bar).

(B) Protein and RNA gel blot analyses of phyB mutant lines. Total proteins or RNA were extracted from 4-d-old etiolated seedlings. Top three lanes: 50 μg each of proteins were served for the detection of PHYA by the monoclonal anti-rye PHYA antibody (mAR07) or PHYB and PHYC by the polyclonal antibody raised against C-halves of PHYB and PHYC proteins, respectively. Bottom two lanes: each lane was loaded with 10 μg of total RNA for the detection of PHYB or PHYC transcripts. Nipponbare (Np) and Norin 8 (N8) were used as controls.

(C) Dilution series of protein extract from Nipponbare were compared with the protein extract from phyB-1 for the PHYC level.

(D) PHYA, PHYB, and PHYC proteins were detected by protein gel blotting of extracts from 4-d-old etiolated seedlings from Nipponbare (WT), phyA-2 (phyA), phyB-1 (phyB), and phyC-1 (phyC) mutants.

(E) Immunoblot detection of PHYC proteins in Nipponbare (WT), phyA-2 (phyA), phyB-1 (phyB), and phyA-2 phyB-1 (phyAphyB) mutants. Four-day-old etiolated seedlings were used for the extraction of proteins. Each lane was loaded with 50 μg of protein for the detection of PHYC protein.
Characterization of Phytochrome Single and Double Mutants

We analyzed the distinct roles of phytochromes in the photomorphogenesis of rice seedlings by comparing single and double phytochrome mutants and Nipponbare (wild type) grown under various light conditions (Figures 2 and 5), including continuous irradiation with red (Rc), FR (FRc), blue (Bc), and white light (Wc) for 9 d. No apparent differences were observed between wild-type and phytochrome mutants grown in the dark (data not shown). As shown in Figure 2A, dark-grown seedlings have long coleoptiles (white arrows) and first leaves (yellow arrows). The most striking feature is the remarkable elongation of second internodes (red arrows), which are hardly measurable in the light-grown seedlings. In principle, light represses the elongation of coleoptiles and first leaves and blocks the internode from elongating (Figures 2B and 2C), but phyA phyB under Rc (Figure 2B), and phyA phyC and phyA phyB double mutants grown under FRc (Figure 2C) showed the typical dark-grown phenotype characterized by the long coleoptiles (white arrows) and the elongated internode (red arrows).

To quantify the differences, we took comprehensive measurements of lengths on the seedlings under Rc and FRc, as well as darkness (Figure 3). Under Rc, the coleoptile elongation was severely inhibited in wild-type, phyA, phyC, and phyA phyC mutants. Coleoptiles of phyB and phyB phyC mutants were similarly longer than those of the wild type but were still greatly reduced in length compared with dark-grown seedlings, and no inhibition was observed in phyA phyB double mutants (Figure 3A, Rc). These results indicate that phyB plays a major role in coleoptile inhibition by Rc and that the role of phyA in coleoptile inhibition is also important, but visible only in the absence of phyB.

Under FRc, coleoptiles of phyA mutants were longer than those of the wild type but still shorter than those of dark-grown seedlings. The phyB mutation did not affect the coleoptile inhibition. The phyC single mutation also showed no effect, but phyA phyC double mutants had long coleoptiles, as long as those of dark-grown seedlings (Figure 3A, FRc). Therefore, when phyA is functional, phyC function is dispensable, but in the absence of phyA, phyC showed a limited effect by partially inhibiting the coleoptile (the difference between phyA and phyA phyC). No inhibition was detected in phyA phyB double mutants, probably due to a significant reduction in the phyC level of these mutants.

First leaves of rice are incomplete and consist mostly of leaf sheaths and poorly developed tiny leaf blades. Thus, the lengths of first leaves represent mostly those of leaf sheaths. The growth of first leaves was also inhibited by Rc and FRc in basically the same manner as observed in coleoptiles (Figure 3B). The inhibitory effects of Rc and FRc appeared less drastic on first leaves than on coleoptiles.

The second internodes of phyA phyB double mutants under Rc and those of phyA phyC and phyA phyB under FRc elongated as much as those of dark-grown seedlings. The other mutants had undetectable internodes when grown under FRc or Rc conditions except for the phyA mutants under FRc, which showed slight but measurable elongation (Figure 3C).

Red Light Effect on the Inhibition of Coleoptile Elongation Was Changed during the Growing Stages

As we mentioned in the previous section, the Rc-mediated growth inhibition of coleoptiles was reduced in phyB mutants, but a substantial inhibition was still observed even in phyB mutants (Figure 3A, Rc). These results suggested that phytochromes other than phyB were also involved in the Rc-mediated inhibition of coleoptile elongation. We performed daily irradiation experiments in order to separate the effects of different phytochromes.

We grew nine sets of wild-type (Nipponbare) and phyB-1 seedlings in darkness for 7 d. Each set was exposed to Rc on a designated day as depicted in Figure 4B, and final coleoptile lengths of these seedlings were compared on day 7 (Figure 4A). The overlaid red curve shows a typical growing curve of coleoptiles of the wild type in the dark condition. Coleoptile elongation

![Figure 2](image-url)
in the dark showed a sigmoid curve and was saturated around day 7.

The Rc irradiation on the first day did not induce any photoinhibition of coleoptile elongation in either wild-type or phyB-1 plants (Figure 4A, condition 1). In the wild-type plants, Rc irradiated on the second day was effective in inhibiting coleoptile elongation (condition 2). The shortest coleoptile length was obtained by the Rc irradiation on the third day (condition 3), which was almost equal to that of seedlings grown under Rc irradiation for 7 d (condition 9). However, the inhibitory effect of red (R) light was significantly reduced in the phyB-1 compared with that of the wild type in conditions 2 and 3, which seemed to cause the elc phenotype. The difference in sensitivity to Rc was no longer significant on and after day 4 (conditions 4 to 8). These results suggest that phyB is essential for detecting R light for inhibition of coleoptile elongation in the early stage of the seedling growth.

It was noteworthy that the mode of response to Rc was changed around day 4 in the wild-type seedlings. Coleoptile lengths just before the Rc irradiation on days 2 and 3 were estimated to be in the range of 2 and 5 mm, but the final lengths were ~16 and 8 mm for conditions 2 and 3, respectively.

Therefore, when the coleoptile length was less than the threshold (before the fourth day), the coleoptile continued growing to a certain extent after the Rc irradiation. However, on and after day 4, coleoptiles stopped growing immediately when the R light was irradiated (tops of bars are on the sigmoidal curve in Figure 4A). These results led us to infer that these differences in the response modes might result from different growth modes of the coleoptile, such as cell division and cell elongation, and that the growth inhibition mediated by phyB was the result of inhibition of cell division.

To define whether our inferences were valid, we measured cell lengths of inner epidermal cells of the coleoptile from the wild-type and phyB-1 seedlings treated as in condition 3 in Figure 4B. The final lengths of coleoptiles were $5.6 \pm 0.18 \text{ mm}$ and $15.4 \pm 0.27 \text{ mm}$, and the average lengths of cells in the middle parts of the coleoptiles were $92 \pm 2.4 \mu m$ and $148 \pm 5.1 \mu m$ for the wild type and phyB-1, respectively (Table 1). Cell division in the coleoptile was unaffected by the mutation because both wild-type and phyB-1 seedlings had approximately the same number

![Figure 4](image-url)

**Figure 4.** Day Dependency of R Light Effect in Photoinhibition of Coleoptile Elongation.

(A) Coleoptile lengths of Nipponbare (open bars) and phyB-1 seedlings (shaded bars) grown in different R light treatment conditions shown in (B). Bars represent relative values based on the average coleoptile lengths of dark-grown seedlings (condition 8). Overlaid red curve shows the growth curve of the coleoptile of Nipponbare seedlings in the complete darkness at 28°C. The means $\pm SE$ obtained from 30 seedlings were plotted.

(B) Schematic drawing of experimental timetable for exposure to Rc. Except for condition 8 (in complete darkness for 7 d) and condition 9 (under Rc for 7 d), each set was exposed to Rc (15 $\mu$mol photons m$^{-2}$ s$^{-1}$) for 1 d in a designated timing.
of cells along the length of the coleoptile (182 ± 7.8 cells and 198 ± 10 cells, respectively), as depicted in Table 1. We also measured the cell lengths and numbers of the coleoptiles just before the Rc irradiation in this condition (2-d-old, dark-grown seedlings). These seedlings had short coleoptiles (<5 mm), yet the cell numbers were the same as those of the seedlings with final lengths (Table 1, rows 1 and 2). Moreover, we measured the same parameters in the seedlings grown in dark for 7 d (condition 8). Cell lengths appeared to be increased 10 times in plants grown for 5 d in the dark, with no detectable change in the numbers of cells (Table 1, row 3). Therefore, the growth of coleoptile can be attributed to the cell elongation, and R light inhibits the cell elongation partly mediated by phyB function.

Blue Light–Induced Declination of Second Leaf

Another significant phenotypical difference was observed in leaf blade angles of second leaves under Bc and Wc (Figures 5A and 5B). We took pictures of seedlings and measured the declination angles of second leaf blades. Bc increased leaf blade declination, and the effects were different among wild-type and phytochrome mutants (Figure 5A). As shown in Figure 5C, declination angles of phyA mutants were the same as those of the wild type, and phyC and phyA phyC mutants showed a slightly greater declination than the wild type. The declination angles were much greater in phyB and phyB phyC mutants and greatest in phyA phyB double mutants (almost at a right angle). These results indicate that phyB and phyC are involved in different ways in the second leaf declination upon perceiving Bc. The phyA also makes a significant contribution to the second leaf declination by Bc, but the strong effect of the phyA mutation is revealed only in the absence of phyB. Under Wc, phyB, phyB phyC, and phyA phyB phyC seedlings showed the same declination angles as in the Bc, but wild-type and phyA, phyC, and phyA phyC phyC mutants did not. These observations suggest that phyB seems to function antagonistically to blue light receptors (maybe cryptochromes) on the leaf blade declination.

**Table 1.** Size and Number of Cells in Inner Epidermal Layers of Wild-Type and *phyB-1* Seedlings

<table>
<thead>
<tr>
<th>Phenotypes of Seedlings</th>
<th>Light Treatmentsa</th>
<th>Cell Size (µm)</th>
<th>Number of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Rp</td>
<td>26.5 ± 1.0</td>
<td>209 ± 9.5</td>
</tr>
<tr>
<td></td>
<td>Rc</td>
<td>92 ± 2.4</td>
<td>182 ± 7.8</td>
</tr>
<tr>
<td></td>
<td>Darkc</td>
<td>326 ± 9.3</td>
<td>222 ± 15</td>
</tr>
<tr>
<td><em>phyB-1</em></td>
<td>Rp</td>
<td>31.5 ± 2.5</td>
<td>218 ± 12</td>
</tr>
<tr>
<td></td>
<td>Rc</td>
<td>148 ± 5.1</td>
<td>198 ± 10</td>
</tr>
<tr>
<td></td>
<td>Darkd</td>
<td>308 ± 12</td>
<td>217 ± 14</td>
</tr>
</tbody>
</table>

Values are averages from 10 to 20 measurements, and standard errors are shown.

aWild-type and *phyB-1* seedlings were grown in darkness and treated with Rc light as in Figure 4B.

bTwo-day-old etiolated seedlings just before the R light treatment in condition 3.

cSeedlings treated as in condition 3.

dSeedlings from condition 8 (i.e., 7-d-old etiolated seedlings).

Figure 5. Second Leaf Blade Declination Induced by Bc and Wc.

(A) and (B) Seedlings were grown under Bc (A) or Wc (B) for 9 d at 28°C. The fluence rates of Bc and Wc are 15 and 40 µmol photons m⁻² s⁻¹, respectively. Wild-type and single and double phytochrome mutants are aligned for comparison from left to right: wild type, *phyA*, *phyB*, *phyC*, *phyA phyC*, *phyB phyC*, and *phyA phyB*. Yellow arrows indicate lamina joints of second leaves. The two pictures are the same magnitude, and scale bars at left sides are 10 mm.

(C) Declination angles are measured from the pictures and shown for wild-type and phytochrome mutants grown under Bc (shaded bars) or Wc (open bars). The means ± se were obtained from 20 to ~50 seedlings.
R/FR Reversibility of Lhcb Gene Expression in the Phytochrome Mutants

We examined the Lhcb gene expression in the rice phytochrome mutants under various light conditions. Rice seedlings grown in the dark for 7 d were exposed to the R light pulse (Rp), FR light pulse (FRp), or to sequential combinations of both Rp and FRp. Fluence of Rp used in this study (1.8 mmol photons m\(^{-2}\)) could induce both VLFR and LFR. Fluence of FRp (9 mmol photons m\(^{-2}\)) was adjusted not only to induce robust VLFR but also to cancel LFR induced by a previously given Rp. After the light treatments, seedlings were kept in darkness for another 3 h and subsequently used for RNA extraction. Lhcb transcript levels were examined by RNA gel blot analysis.

The results (Figure 6) indicated that the Lhcb gene was highly induced by a single Rp treatment in wild-type seedlings (lane 1), and ～70% of the total induction was canceled by a subsequent FRp. The diminished level was comparable with the level observed in seedlings exposed to a single FRp, which corresponds to VLFR. Thus, the difference between Rp- and FRp-mediated Lhcb gene expression is thought to be a contribution of LFR. Unexpectedly, phyB mutants also displayed clear R/FR reversibility in the Lhcb transcript levels (lane 3), which was indistinguishable from that of the wild type (lane 1). All phyB alleles isolated in this study also showed similar results (data not shown). These results show that a phytochrome(s) other than phyB regulates the R/FR-reversible LFR in phyB mutants. To identify the phytochrome(s) responsible for this effect, single and double mutants for other phytochromes were examined for the induction of Lhcb genes.

As in lane2, phyA mutants showed a clear R/FR reversibility but lost the induction of Lhcb genes by a FRp, which is consistent with the report that phyA acted as a major photoreceptor for VLFR (Takano et al., 2001). phyC mutants showed the R/FR reversibility similar to phyB mutants (lane 4). Deficiency of either phyB or phyC did not affect amplitudes of the inductions by LFR and VLFR. The induction patterns of phyA phyC double mutants (lane 5) were similar to that of phyA single mutants, but the induction levels mediated by the LFR were reduced. The most unexpected result was the display of clear R/FR reversibility by phyB phyC double mutants (lane 6), where phyA is the only active phytochrome species. These observations indicate that phyA can mediate the R/FR-reversible LFR on the regulation of Lhcb gene expression in rice etiolated seedlings. The phyA phyB double mutants did not show any induction with either Rp or FRp in our experimental conditions. Quite similar results have been reported in the Arabidopsis phyA phyB double mutant (Reed et al., 1994).

We also examined Ribulose bisphosphate carboxylase/oxygenase small subunit (RbcS) gene expression. The regulatory patterns were basically the same as those of Lhcb, but the reversibility was not so clear because of the higher basal level of RbcS gene expression in the dark (data not shown).

Continuous Light–Induced Gene Expression in the Phytochrome Mutants

In an early study of rice phyA mutants (Takano et al., 2001), we recognized that Frc induced Lhcb and RbcS gene expression even in the phyA mutants, although the expression level was very low. We anticipated that such induction might result from phyC function. To verify this hypothesis, we determined the expression levels of Lhcb and RbcS genes in the seedlings of wild-type and phytochrome single and double mutants grown in darkness or under Frc or Rc. As shown in Figure 7A, Lhcb and RbcS genes were induced by Frc in the wild type, phyB, phyC, and phyB phyC at the same level and were less induced in phyA mutants. However, phyA phyB and phyA phyC double mutants did not show any induction of these genes under Frc compared with the dark condition. The expression patterns between phyA and phyA phyC mutants can be simply compared, which leads to the conclusion that the residual response to Frc in the phyA mutant is diminished when coupled with phyC mutation. These results indicate that phyC has the ability to perceive Frc for inducing Lhcb and RbcS gene expression. However, we need to be careful to interpret the results of the phyA phyB mutant, considering that the phyB mutation causes the reduction of phyC.

Under Rc (Figure 7B), Lhcb and RbcS genes were equally induced in phyA, phyC, and phyA phyC mutants at the same level as in the wild type. The phyB mutation reduced the induction levels of these genes, but phyB phyC double mutants showed induction at the same level as phyB mutants, indicating that phyB is a dominant phytochrome responsible for Lhcb and RbcS gene induction by Rc and that phyC makes little contribution to the

### Figure 6. Induction of Lhcb Gene Expression by R and/or FR Pulses in Etiolated Rice Seedlings.

Rice seedlings from Nipponbare (WT) and phytochrome single (phyA, phyB, and phyC) and double (phyA phyC, phyB phyC, and phyA phyB) mutants were grown in complete darkness for 7 d (D) and then treated with a single Rp (R), a FRp immediately after an Rp (R/FR), a train of Rp-FRp-Rp (R/FR/R), or a single FRp (FR). Seedlings were harvested 3 h after the pulse irradiation, and Lhcb gene expression was analyzed by RNA gel blot hybridization. rRNA was stained by methylene blue with the blot of wild type as a quantity control.
induction. In phyA phyB double mutants, the inductions of Lhcb and RbcS genes by Rc were completely diminished. These results can lead to the interpretation that phyA perceives Rc for inducing Lhcb and RbcS gene expression because phyC has no effect on the Rc-mediated response.

Flowering Times of the Phytochrome Mutants

We examined flowering times of phytochrome mutants under natural daylength (in the paddy field) and short-day (SD) conditions (in the growth chamber). Because daylengths during this cultivation were >13 h, our natural daylength condition was considered equivalent to LD conditions. Under natural daylength (Figure 8A), the wild type (Nipponbare) flowered in ~97 d, and phyA mutants did the same as the wild type, while phyB and phyC mutants were ~12 d earlier than the wild type. Both alleles of phyB mutants with Nipponbare background (phyB-1 and phyB-2) showed the same early flowering phenotype. The phyB mutants with Norin 8 background also flowered earlier than Norin 8 by ~2 weeks. It was noteworthy that the weak allele, phyB-3, showed an intermediate phenotype; it flowered earlier than Norin 8 but later than the other null alleles. We also determined the flowering times of the selected PHYC-antisense transgenic lines. Under LD conditions, these PHYC-antisense lines flowered earlier than Nipponbare to the extent that is similar to the phyC mutant (X. Xie and M. Takano, unpublished results). These observations clearly indicate that phyB and phyC are involved in the control of flowering time to delay in response to LD conditions.

Double mutations showed synergistic or redundant effects of individual phytochromes depending on the combinations. phyB phyC double mutants flowered as early as phyB or phyC single mutants, suggesting that phyB and phyC have the same effect on the flowering-time determination under natural daylength. Interestingly, phyA phyB and phyA phyC double mutants flowered dramatically earlier than any single mutants under natural daylength. The floral initiations in phyA phyB and phyA phyC double mutants were ~22 d ahead of phyB or phyC monogenic mutants, while phyA monogenic mutants flowered at approximately the same time as the wild type. These observations indicate that phyA mutation alone does not affect the flowering time much, but in the phyB or phyC mutant background, phyA mutation makes a big contribution in determining the flowering time in LD conditions.

The floral induction of Nipponbare (wild type) was significantly enhanced under SD conditions (10 h of light/14 h of dark [10L/14D]; Figure 8B) compared with LD conditions. While phyA mutants flowered slightly later than the wild type, phyB mutants came into flower still earlier than the wild type even in the SD conditions. The flowering time of phyC mutants was approximately the same

Figure 7. Induction of Lhcb and RbcS Genes by FRc or Rc in Wild-Type and Phytochrome Mutant Seedlings.

Nipponbare (WT) and phytochrome single (phyA, phyB, and phyC) and double (AB, phyA phyB; AC, phyA phyC; BC, phyB phyC) mutants were grown in the dark (D) or under FRc [FR in [A]] or Rc (R in [B]) for 4 d. Lhcb and RbcS gene expression was analyzed by RNA gel blot hybridization. rRNA was stained by methylene blue with the same blot as a quantity control.

Figure 8. Comparison of Flowering Times between Wild-Type and phyB Mutants under Natural Daylengths and SD Conditions.

(A) and (B) Nipponbare (WT) and phytochrome single (phyA, phyB, and phyC) and double (phyA phyC, phyB phyC, and phyA phyB) mutants were grown in the paddy field (A) or in a growth chamber set as SD (10L/14D; [B], open bars) or LD (14L/10D; [B], shaded bars), and their flowering times were measured (natural daylength).

(C) Wild-type (open bars) and phyB mutants (hatched bars) were grown in a growth chamber set as SD (normal, 10L/14D; severe, 8L/16D) or SD (10L/14D) plus EDO-FR conditions. The means ± se obtained from 20 plants are displayed.
as that of the wild type; moreover, the flowering times of phyA phyC and phyB phyC double mutants were the same as those of phyA and phyB single mutants, respectively. These results indicate that phyC has no significant effects on the floral induction under SD conditions. Unexpected results were obtained from phyA phyB double mutants, which flowered significantly later than the wild type under SD conditions. To evaluate the differences of the flowering times between LD and SD conditions more precisely, we grew the double mutants in the same growth chamber with LD conditions (Figure 8B, shaded bars). The floral induction in the phyB phyC double mutants was greatly enhanced with the reduction in daylength, and this behavior matches with that of a phyB single mutant. Interestingly, phyA phyC double mutants showed the same flowering times in both SD and LD conditions. Furthermore, the flowering time of the phyA phyB double mutants was earlier in LD than in SD conditions.

To examine phyB function in floral induction under SD conditions, we extended the dark period of SD (Figure 8C). In severe SD conditions (8 h of light/16 h of dark [8L/16D]), the wild type flowered even earlier than in the normal SD, and the difference of flowering times between the wild type and phyB mutants became smaller. These observations suggest that dark reversion of phyB is involved in the flowering time determination. Thus, we gave end-of-day FR (EOD-FR) light treatments to both wild-type and phyB mutants grown under SD conditions (10L/14D) to see the involvement of the Pfr form of phyB in darkness on the floral initiation. As shown in Figure 8C, the EOD-FR treatments induced early flowering in Nipponbare that was as early as that of phyB mutants. These observations suggest that the Pfr form of phyB remaining over the dark period is involved in the repression of flowering in the SD (10L/14D) conditions.

DISCUSSION

Isolation of Rice phyB Mutants

We screened γ-ray–mutagenized rice seedlings for phyB mutants that showed the elc phenotype under Rc. As a result, we have isolated five alleles of phyB mutants, all of which had deletions (phyB-2, phyB-3, phyB-4, and phyB-5) or an insertion (phyB-1) of nucleotides in the PHYB genes (see Supplemental Figure 1 online). The deletions or insertion created in-frame stop codons in the middle of the gene, resulting in immature translation stops in the phyB-1, phyB-2, phyB-4, and phyB-5 alleles. PHYB protein was not detectable in these mutants based on the protein gel blot analysis, suggesting that the mutants have null mutations in PHYB genes (Figure 1B). On the other hand, phyB-3 appears to carry a partial loss-of-function mutation because the Rc-mediated inhibition of coleoptile elongation was more pronounced in phyB-3 than in other phyB mutants, and early flowering phenotype was partially suppressed (data not shown). Sequence and protein/RNA gel blot analyses also support this conclusion. The 33-bp deletion caused the loss of the last nine amino acids, including the stop codon in the PHYB open reading frame, and added a new C terminus of 62 amino acids (see Supplemental Figure 1 online). The extension does not seem to disarm the PHYB protein completely. Actually, mRNA expression level of the PHYB gene in phyB-3 was comparable to that in the wild-type plants, and a band with shifted mobility was detected with reduced intensity in the phyB-3 by protein gel blot analysis (Figure 1B).

PHYC Proteins in phyB Mutants

Hirschfeld et al. (1998) reported that PHYC protein levels were greatly reduced in the Arabidopsis phyB mutants. We also detected a significant reduction of PHYC proteins in the rice phyB mutants, but the transcript levels were not affected by the phyB mutation (Figure 1B), suggesting that the phenomenon is likely to be inherent in the PHYC protein. Recently, comprehensive communoprecipitation assays for Arabidopsis phytochromes have shown that phyC forms a heterodimer with phyB in planta (Sharrock and Clack, 2004). In rice, we also found that PHYC was communoprecipitated with PHYB (X. Xie and M. Takano, unpublished results). Thus, deficiency of phyB could cause a reduction in the stability of phyC in rice as well.

Phenotypical Features of Rice Phytochrome Mutants

Under Rc, rice phyB mutants showed significant inhibition of coleoptile elongation (Figure 3A). The difference in final lengths between wild-type and phyB mutants was found to result from the absence of functional phytochromes during the early stage of germination in the phyB mutants (Figure 4A; discussed below). The inhibitory effect was not influenced in the phyB phyC double mutants but was completely canceled in the phyA phyB double mutants, which showed a completely etiolated phenotype under Rc (Figures 2B and 3, Rc). These observations indicate that both phyA and phyB equally contribute to the perception of Rc for the coleoptile inhibition and that phyC has no effect on it. Under FRC, phyA phyC double mutants looked just like dark-grown seedlings (Figure 2C). Moreover, anatomical measurements (Figure 3, FRC) as well as light-induced gene expression patterns (Figure 7A) of phyA phyC double mutants were indistinguishable from those of dark-grown seedlings. These results indicate that the phyA phyC double mutant cannot detect FRC as light signals and leads to the conclusion that not only phyA but also phyC can perceive FRC. Although phyA is considered a predominant photoreceptor for FRC and phyC function is usually dispensable when phyA is functional, several phenotypes, such as second leaf sheath inhibition (data not shown), indicate the independent involvement of phyC in the FRC perception.

FR light had no effect on phyA phyB double mutants either (Figures 2C and 3, FRC). Because phyC levels are greatly reduced by the phyB mutation (Figure 1B), the remainder of the phyC would not be enough to perceive FRC for the growth inhibition. Another plausible explanation is that the residual phyC in the phyB mutant has no detectable activity, and in such a situation only phyB phyC heterodimers could respond to FRC. We consider the latter to be more applicable because, first, the phyA phyB double mutant showed a totally dark phenotype even after 9 d of FRC exposure (Figures 2C and 7A) and, second, the phyB mutant behaves in the same way as the phyB phyC double mutant for all the deetiolation responses examined.

Recently, two research groups have reported the isolation of Arabidopsis phyC (Franklin et al., 2003; Monte et al., 2003).
Characterization of these phyC mutants indicated that phyC was involved in the response to Rc but not to FRc in the seedling deetiolation process in Arabidopsis. Therefore, the photosensory specificity phyC differs between Arabidopsis (similar to phyB and phyD) and rice (more like phyA).

The declination of second leaf blades under Wc conditions was also a remarkable phenotype of phyB mutants (Figures 5B). Because Bc caused the leaf declination in even wild-type seedlings (Figure 5A), we believe that blue light promotes the declination via the cryptochrome function and that phyB behaves antagonistic to cryptochromes. The Bc effects were different in the different phytochrome mutants probably because phytochromes also perceived Bc to antagonize the declination with different efficiencies; phyB was most effective and phyC was next. Here again, phyA single mutants were indistinguishable from the wild type, but phyA phyB double mutants showed drastic declination, indicating that phyA partially complements the phyB function when phyB is missing.

Contribution of Different Phytochromes on the Coleoptile Inhibition

One of the typical phenotypes of phyB mutants was less sensitivity to R light on the inhibition of coleoptile elongation, but the photoinhibition was still observed in phyB mutants (Figure 3A, Rc). We found two phases with different responsiveness to R light by analyzing the time-dependent effectiveness of the wild type to the R light treatment (Figure 4). In the first phase, the coleoptile continued growing to a certain extent after the irradiation when R light was irradiated during the early stage of germination (for the first 3 d). However, on and after the fourth day (second phase), the growth of the coleoptile was immediately inhibited by the R light irradiation. This observation reproduced the results reported by Pjon and Furuya (1967), indicating that the response mode of coleoptile to R light had changed during the development. We anticipated that such differences could result from the different growth modes of coleoptiles during the development, but measurements of cell number revealed that even in the early stage, the growth of coleoptile is attributed to the cell elongation, which suggests that mechanisms of cell elongation might be changed during the coleoptile development.

Observations on the second and third days revealed that wild-type seedlings responded well to R light in inhibiting coleoptile elongation, whereas the response of phyB-1 was meager. On and after day 4, however, the sensitivity to R light observed in the phyB-1 was nearly comparable to that in the wild type (Figure 4A). These findings suggest that phyB acts as a major photoreceptor for perceiving R light in the early phase of germination; however, in the later stage, other phytochromes were also able to perceive R light to inhibit the elongation, and phyB function was no longer indispensable. Therefore, in the first few days after germination, phyB mutants have no active phytochromes. As a result, coleoptiles continue to grow even under Rc during this period, and the difference of growth increment during this period causes the elc phenotype in phyB mutants. Similar observations have been reported in the hypocotyl growth inhibition of tomato phyB1 mutants (van Tuinen et al., 1995b) and the light-regulated germination of Arabidopsis seeds (Shinomura et al., 1996). van Tuinen et al. (1995b) found that the tomato phyB1 mutant was insensitive to Rp only during the first 2 d upon germination, and the inhibitory effect of Rp on hypocotyl growth inhibition was retained thereafter. In Arabidopsis, the phyB-mediated germination was detectable only 3 h after immersion of the seeds into water. However, an incubation of 48 h was necessary to detect phyA-mediated germination response at a maximum level. Therefore, the photoinhibition of coleoptile growth seems to be mediated by sequential activation of light perception systems in etiolated rice seedlings.

Light-Regulated Lhcb Gene Expression in Phytochrome Mutants

Rice etiolated seedlings displayed clear R/FR reversibility in the induction of Lhcb genes (Figure 6, lane 1). In wild-type Arabidopsis seedlings, R light induced Lhcb gene expression, but the induction was not apparently reversed by subsequent FR light because phyA-specific induction of Lhcb gene was photoirreversible (Hamazato et al., 1997; Cerdan et al., 1999). Therefore, the photoperception mode of phyA for Lhcb gene expression may be different in rice and Arabidopsis. Such an outcome was supported by unexpected results from rice phyB mutants. All phyB mutants isolated in this work showed clear R/FR reversibility in the expression of Lhcb genes (representative data shown in Figure 6, lane 3). The level of phyC was also greatly reduced in phyB mutants (Figure 1B) so that phyC was not likely to be involved in the R/FR-reversible LFR in phyB mutants. As a matter of fact, the phyB phyC double mutants also showed clear R/FR reversibility (Figure 6, lane 6). In this mutant, only phyA is an active phytochrome; yet, not only did Rp and FRp induce Lhcb expression, but also the Rp-induced expression was reversed by a subsequent FRp, which means that rice phyA responds to R light in two ways (VLFR and LFR).

There are several reports to indicate that phyA is involved in the R/FR-reversible LFR. It has been known that blue light–induced phototropic curvature is enhanced by preirradiation of Rc in a fluence-dependent manner. Stowe-Evans et al. (2001) have demonstrated that the phototropic enhancement is primarily a phyA-dependent R/FR-reversible LFR in Arabidopsis. Long and lino (2001) examined light-dependent osmoregulation in stem protoplasts from pea (Pisum sativum) phytochrome mutants and obtained results suggesting that phyA could mediate the swelling responses in an R/FR-reversible manner. Since these responses were observed under Rc conditions, light-stable phyA may be in charge of the R/FR-reversible LFR. Thus, it can be postulated that there are two types of phyA molecules with different photoperception properties and that the ratio of these two types varies depending on the physiological responses. On the other hand, results from phyA phyC mutants indicated that rice phyB mediated only R/FR-reversible LFR (Figure 6, lane 5), which is consistent with the results obtained from the characterization of phyB mutants in Arabidopsis (Hamazato et al., 1997).

Flowering Time of Phytochrome Mutants

The rice phyB mutants flowered earlier than the wild type in both LD and SD conditions (Figure 8), which was expected based on
the data published for the sorghum (*Sorghum bicolor*) phyB mutant (Childs et al., 1997). Thus, the inhibitory effect of phyB on the floral initiation under both SD and LD is observed commonly in flowering plants irrespective of LD or SD plants (Halliday et al., 1994; Childs et al., 1995). Moreover, an extended dark period (8L/16D) or EOD-FR treatments under SD (10L/14D) conditions hastened floral initiation of the wild type, resulting in the same flowering time as of phyB mutants (Figure 8C). Such manifestations of phyB mutants in the SD conditions are well explained by the external coincident model proposed by Izawa et al. (2002), in which Pfr forms of phytochromes modulate the PHOTOPERIODIC SENSITIVITY1 (SE1) function to be a repressor of FLOWERING LOCUS T (FT)-like gene expression. Applying their model to our results obtained in the SD (10L/14D) conditions, residual amounts of Pfr forms of phyB are able to interact with SE1, whose expression level peaks at midnight (Izawa et al., 2002), resulting in a certain degree of delay of flowering compared with *phyB*-1. However, extending the dark period or photoconversion of Pfr to Pr form removed the functional phyB and made SE1 fully active to promote flowering.

The flowering date of *phyC* mutants was ~13 d earlier than that of the wild type and was as early as that of *phyB* in natural daylength conditions (Figure 8A). Furthermore, *phyB phyC* double mutants flowered at the same time as *phyB* or *phyC* monogenic mutants (Figure 8A). In SD conditions, however, *phyC* mutants flowered at the same time as the wild type, and the flowering time of *phyB phyC* double mutants was the same as that of *phyB* monogenic mutants. These observations indicate that phyC, along with phyB, is required for delaying floral initiation in response to the LD signals (suppressive conditions in rice) but has no effect on flowering time in SD conditions (inductive conditions) with or without phyB.

The *phyA* monogenic mutants show the same flowering time as the wild type under natural daylength conditions. However, in the *phyB* or *phyC* mutant background, the *phyA* mutation greatly accelerated the flowering so that *phyA phyB* and *phyA phyC* double mutants flowered 26 d earlier than *phyB* or *phyC* monogenic mutants (Figure 8A). Thus, as long as both phyB and phyC function normally, phyA has little effect on the determination of the flowering time, but in the absence of phyB or phyC, the contribution of phyA to delay flowering is greatly increased under LD conditions. These results suggest that phyA acts at different points in the LD pathway from the phyB and phyC to suppress the floral induction in response to the LD photoperiod. Under SD conditions, the *phyA* monogenic mutants showed slightly late flowering compared with the wild type. The flowering time of *phyA phyC* double mutants was the same as that of *phyA* monogenic mutants, indicating that phyC has no function for promoting flowering in the *phyA* background as well.

When three double mutants are compared with each other for their responses to different photoperiods, all appear to be different in their responses, as shown in Figure 8B. The *phyA phyB* double mutant, which almost completely lacks all three phytochromes, flowered significantly later than the wild type or any of the other mutants under SD conditions and, as a result, flowered earlier in the LD than in the SD conditions. The *phyA phyC* double mutant, where phyB is the only active phytochrome, showed the same early flowering as the wild type under SD conditions but also flowered early under LD conditions. On the other hand, the *phyB phyC* double mutant, in which only phyA is functional, flowered early in response to the SD conditions and flowered late under LD conditions. These results suggest that in rice, the light signals mediated by the phytochromes promote flowering in response to the SD conditions, while delaying the flowering under LD conditions. Among the phytochromes, phyB alone seems to be involved in the SD signal transduction but not in the LD signaling, while phyA mediates the signals in response to both photoperiods.

We summarized individual and cooperative effects of phytochromes on the flowering time determination under SD and LD conditions in rice and compared them with those in *Arabidopsis* (Figure 9). LD conditions in rice correspond to SD conditions in *Arabidopsis* for suppressing flowering, and SD conditions in rice and LD conditions in *Arabidopsis* have the same effect of promoting flowering.

In both rice and *Arabidopsis*, monogenic mutation in phyB or phyC causes early flowering under inadequate photoperiod for the plants. The contributions of phyB and phyC are the same in rice, while phyB function is dominant over phyC in *Arabidopsis* (Monte et al., 2003). The phyA functions, especially in combination with other phytochromes are quite different between rice and *Arabidopsis*.

**Figure 9.** Two-Way Table of Flowering Time for Single and Double Phytochrome Mutants under SD and LD Conditions in Rice and *Arabidopsis*.

Value of each cell represents the effect of a single or a double mutation indicated by a combination of the corresponding row (left) and column (top). Combinations of the same mutation in row and column indicate the results for the single mutants (e.g., a cell with row phyA and column phyC indicates a value from phyA single mutants). To facilitate the comparison of data in one row, results from double mutants are duplicated in the table (e.g., *phyA phyB* and *phyB phyA* are the same data). Flowering time of each mutant is compared with that of the wild type in the same photoperiod. Figures in the cells are relative values of flowering time based on the wild type and visualized by arrows at the left. Horizontal arrows, same as the wild type; downward arrows, earlier than the wild type; upward arrows, later than the wild type. The number of arrows represents the extent of the effect. The values of *Arabidopsis* are calculated from the data reported by Reed et al. (1994), Neff and Chory (1998), and Monte et al. (2003). Photoperiods with the same effects on the flowering of each plant (LD for rice and SD for *Arabidopsis* are suppressive, and SD for rice and LD for *Arabidopsis* are inductive) are aligned.
and *Arabidopsis*. In rice, the *phyA* mutation greatly accelerated flowering in the *phyB* and *phyC* background, but no significant effect of the *phyA* mutation was observed in the *phyB* and *phyC* background in *Arabidopsis*.

Under the inductive conditions, monogenic mutation of each phytochrome showed essentially the same effect in both rice and *Arabidopsis*. The *phyA* mutation delays, the *phyB* accelerates, and the *phyC* has no effect on the flowering, but the double mutations caused different responses. In *Arabidopsis*, the flowering time of *phyA* *phyB* double mutants was intermediate between those of single mutants, demonstrating additivity or antagonism between these phytochromes (Neff and Chory, 1998). The *phyA* *phyC* double mutant caused late flowering, suggesting that *phyA* and *phyC* play a redundant role in promoting flowering in LD conditions (Monte et al., 2003). It has been reported in *Arabidopsis* that *phyA* directly regulates *FT* expression by affecting CONSTANS (CO) function (Yanovsky and Kay, 2002). In this case, the *phyA* function is to accelerate the flowering by making the light signal meet high levels of CO mRNA in LD conditions (the so-called external coincidence mechanism), and the early flowering can be observed under FR-rich light conditions. In the case of rice, *phyA* suppresses flowering under LD conditions, and the effect does not depend on light quality. These observations suggest that the role of *phyA* in flowering differs between *Arabidopsis* and rice, at least under LD conditions. The external coincidence model, which explains the photoperiodic regulation of flowering time in *Arabidopsis*, cannot be simply applied to SD plants such as rice. Currently, for rice, all phytochrome mutants as well as several near iso-specific lines with mutated flowering time genes, such as *heading date 1* (*hd1*) and *hd3a*, are available (Yano et al., 2000; Kojima et al., 2002). Comparing the flowering times of these mutants and their crossbreds in various photoperiods will provide us some idea about the mechanism of flowering time determination in rice.

The work presented here is part of our continuing effort to understand the mechanism of photoperception by phytochromes in rice. We have isolated all phytochrome mutants (*phyA*, *phyB*, and *phyC*) from rice and also produced double mutants. Analyzing these mutants gave us clearer pictures about the individual function of each phytochrome as well as interactions among them. We found that the photoperception mode of *phyC* differs between rice (mainly perceiving FR) and *Arabidopsis* (R receptor; Hirochika, 1999). DNA isolated from the mutant plants were organized as superpools, which were designated as mutant panels and were sequenced to confirm the insertion of *Tos17-LTR* by PCR for *phyC* mutants in this study has led us to conclude that the function of *phyB* is somewhat universal among flowering plants. We also have uncovered the functions of rice phyA (R/FR-reversible LFR in the *Lhcb* gene expression), which were previously undetected even by the analysis of *phyA* mutants (Takano et al., 2001), and have not been reported in *Arabidopsis* or tomato *phyA*. In most plants investigated to date, *phyA* mutants have not shown remarkable phenotypes except for in the pea, where *phyA* mutants show a highly pleiotropic phenotype (Weller et al., 1997) or light-dependent osmoregulation (Long and lino, 2001). Thus, in contrast with *phyB*, *phyA* may have distinct additional functions depending on the plant species. We hope that these results from rice phytochrome mutants will serve as references to evaluate the results from *Arabidopsis*.

**METHODS**

**Monochromatic Light Sources**

Unless otherwise described, we used R light-emitting diode panel (Model LED-R; EYELA), FR light-emitting diode panel (Model LED-FR; EYELA), and blue light-emitting diode panel (Model LED-B; EYELA) for monochromatic light sources. The FR emitting diode panel was equipped into a filter box with one layer of acryl cutoff filter (KYOWALITE PG, SP-60-3K 202, thickness ¼ mm; Kyowa Gas Chemical). White light was supplied by white fluorescent tubes (FL40SN-SDL; NEC).

**γ-Ray Mutagenesis and Screening for eic Mutants**

Dry dormant seeds of rice (*Oryza sativa* cv Nipponbare) were exposed to 250 Gy of γ-ray emitted from 60Co, at a dose rate of 12.5 Gy h\(^{-1}\). The M2 seeds were used for screening for eic mutants. Thirty-seven lines of early flowering mutants of *O. sativa* cv Norin 8 were also used to screen for eic mutants. These early flowering mutants were generated by chronic γ-ray irradiation with dose rates ranging between 3 and 6 Gy d\(^{-1}\).

Seeds were surface sterilized and sown onto 0.4% (w/v) agar. Seedlings were grown under 15 μmol photons m\(^{-2}\) s\(^{-1}\) of Rc irradiation at 28°C for 7 d. We selected seedlings displaying the eic phenotype and replanted them in soil to obtain seeds for further studies.

**Sequencing of eic Mutant Alleles**

A series of oligodeoxynucleotide primers corresponding to the cDNA sequence of rice *PHYB* (AB109892) were synthesized. These were used to amplify segments of the *PHYB* gene from genomic DNA by PCR amplification. PCR products from the wild type and the different mutant alleles were sequenced and compared with detect the mutation sites.

**Screening for phyC Mutant**

Large populations of rice (*O. sativa* cv Nipponbare) mutants generated by means of *Tos17*-mediated mutagenesis were made available by the Laboratory of Gene Function at the National Institute of Agrobiological Sciences. Details of mutagenesis with *Tos17* have been described (Hirochika, 1999). DNA isolated from the mutant plants were organized as superpools, which were designated as mutant panels and were efficiently screened by PCR (Takano et al., 2001) using primers specific to *PHYC* and *Tos17*. To eliminate nonspecific amplifications, we designed additional *PHYC*-specific and *Tos17*-specific primers just downstream of each primer for nested PCR. The primer combinations that provided the correct amplification were *PHYC*-BR (5’-GTGTGAGCCA-GACCATCAACC-3’)/*Tos17-LTR4* (5’-GCTGACATGCGCACAATCT-3’) for the first amplification and *PHYC*BR1 (5’-CACTGTCTCCATCATCCATCC-3’)/*Tos17-LTR5* (5’-CAGTACATTAGCTTGATATATATAAATTA-3’) for the nested amplification. The amplified DNA fragments were cloned and sequenced to confirm the insertion of *Tos17*.

**Constitution of Double Mutants**

To obtain *phyA* *phyB*, *phyA* *phyC*, and *phyB* *phyC* double mutants, *phyA*-2 or *phyA*-4, *phyB*-1, and *phyC*-1 mutants were crossed with each other. *phyA*-4 is a new allele for the *PHYA* mutation with Nipponbare background (Takano et al., 2005). In the *phyA*-4 mutant, *Tos17* is inserted in the middle of the second exon of the *PHYA* gene (1724th nucleotide in the sequence of *PHYA* cDNA, accession number AB109891), resulting a null mutation. The F2 segregants were examined for their genotypes by DNA gel blot hybridization for *phyA* and *phyC* or by PCR for *phyB* and *phyC*. To distinguish between wild-type and the *phyA* or *phyC*-1 mutant alleles, genomic DNAs
isolated from the F2 segregants were digested by PstI for PHYA or XhoI for PHYC and subjected to DNA gel blot hybridization probed with a PHYA- or PHYC-specific sequence. The probe sequences were amplified by PCR from cDNAs using primer pairs of PHYA-IF (5'-AGCAAGCCTCAGCAGAAT)/PHYER (AGGATGAAGTGGACATGCC-3') for PHYA and PHYC-AF (5'-CATGAAAGAAAGTGCGG-3')/PHYC-DR (5'-CATACCGTAAGCGGGAAGGGAC-3') for PHYC. The insertion of Tos17 into PHYA or PHYC gene produces the restriction fragment length polymorphism. The wild-type PHYA allele gives a 4.3-kb band, while the Tos17-inserted PHYA allele produces a 6.9-kb band because the inserted Tos17 sequence has a single PstI site. Similarly, a 3.8-kb band and a 7.5-kb band were obtained by XhoI digestion in the wild-type and Tos17-inserted PHYC alleles, respectively. The phyB-1 mutation is the result of one nucleotide insertion, which creates a new restriction site of NalI (New England Biolabs). To detect this mutation, two PCR primers (PHYBF1, 5'-GGGTTCATTGCTATGCCTTGT-3', and PHYBFR2, 5'-TTGCCATTTGC-TTCTCAAC-3') were designed to amplify the 1-kb fragment, which has a single nucleotide insertion in the middle of the sequence. Thus, the digestion of the amplified fragment with NalI gives two bands for the wild-type allele and three bands for the mutant allele.

The F2 segregants that had homozygous mutant alleles for both genes, PHYA and PHYB, PHYA and PHYC, or PHYB and PHYC, were selected and propagated for the further analyses.

Growth Conditions for the Flowering Time Measurements

Seeds of Nipponbare and the mutant lines were sown on May 10, 2004, and seedlings were transplanted on June 4 in an irrigated rice field. Plants were grown under natural field conditions, and the heading (flowering) date was monitored for the appearance of the first panicle. Nipponbare and mutant lines were also grown in a growth chamber in SD (light cycle, 10h of light/14h of dark; 28°C by day/23°C by night) or LD (light cycle, 14h of light/10h of dark; 28°C by day/23°C by night) conditions. One of the SD set growth chambers has FR light sources to examine EOD-FR effects. Plants were given FR light immediately after cessation of metal halide lamps of the chamber. Transgenic lines and Nipponbare as control were grown in a growth chamber under the SD or the LD photoperiods as above. Light source was metal halide lamps (390 μmol photons m⁻² s⁻¹).

RNA Analysis

For mRNA detection from each phytochrome mutant, seedlings of mutants and Nipponbare were germinated and grown at 28°C for 4 d in complete darkness, and total RNA was isolated by shoot parts from seedlings with the RNeasy plant mini kit (Qiagen). For the light-induced gene expression experiments, seedlings of mutants and Nipponbare were grown in darkness or under continuous FR light (29 μmol photons m⁻² s⁻¹) for 4 d and then harvested. Total RNAs extracted with the RNeasy plant mini kit were separated by 0.8% agarose gels and transferred to Hybond N⁺ (Amersham Bioscience). Lhcb (LHCP II, accession number D0062) and RbcS (accession number X07515) cDNAs from rice were provided by N. Yamamoto (Ochanomizu University, Tokyo, Japan).

Protein Analysis

To raise PHYB- and PHYC-specific antibodies, we expressed the 3'-half moiety of PHYB (from 2768 to 3714, AB108982) or PHYC cDNA (from 2812 to 3710, AB18442) in the Escherichia coli protein expression system (pET16b; Novagen) as His-tagged protein and purified the protein by BD TALON metal affinity resins (BD Biosciences). Rabbits were immunized with the C-terminal half of PHYB (from 857 to 1171 of the amino acid sequence) or PHYC protein (from 839 to 1137). The specificity of the antibody was confirmed by protein gel blot analysis, which showed absence of band in the phyB or phyC mutants. The anti-PHYA antibody used in this study was the anti-rye PHYA monoclonal antibody (mAR07) from the monoclonal antibody stock at the Hitachi Central Laboratory. Immunological analysis was performed as by Takano et al. (2001).

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 Rc, FrC, Bc, or Wc conditions at 28°C for 9 d. Fluence rates were 15 μmol photons m⁻² s⁻¹ for Rc, FrC, and Bc and 40 μmol photons m⁻² s⁻¹ for Wc. The seedlings were removed after 9 d, and their images were captured (e.g., Figures 2 and 5). Lengths and angles of seedling parts were measured from the images.

For measuring cell sizes, inner epidermal layers were peeled and directly observed under a microscope.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AB108981 (PHYA), AB108982 (PHYB), AB18442 (PHYC), D0062 (Lhcb), and X07515 (RbcS).

Supplemental Data

The following material is available in the online version of this article.

Supplemental Figure 1. DNA Sequence Analysis of PHYB Gene Regions in elc Mutants.

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REFERENCES


