Isolation and Characterization of Rice Phytochrome A Mutants

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To elucidate phytochrome A (phyA) function in rice, we screened a large population of retrotransposon (Tos17) insertion mutants by polymerase chain reaction and isolated three independent phyA mutant lines. Sequencing of the Tos17 insertion sites confirmed that the Tos17s interrupted exons of PHYA genes in these mutant lines. Moreover, the phyA polypeptides were not immunochemically detectable in these phyA mutants. The seedlings of phyA mutants grown in continuous far-red light showed essentially the same phenotype as dark-grown seedlings, indicating the insensitivity of phyA mutants to far-red light. The etiolated seedlings of phyA mutants also were insensitive to a pulse of far-red light or very low fluence red light. In contrast, phyA mutants were morphologically indistinguishable from wild type under continuous red light. Therefore, rice phyA controls photomorphogenesis in two distinct modes of photoperception—far-red light–dependent high irradiance response and very low fluence response—and such function seems to be unique and restricted to the deetiolation process. Interestingly, continuous far-red light induced the expression of CAB and RBCS genes in rice phyA seedlings, suggesting the existence of a photoreceptor(s) other than phyA that can perceive continuous far-red light in the etiolated seedlings.

INTRODUCTION

Light is one of the most important environmental stimuli that plays a pivotal role in the regulation of plant growth, development, and metabolic activities. The perception of environmental light by plants is achieved by a family of plant photoreceptors that includes the phytochromes (Neff et al., 2000), cryptochromes, and several others (Briggs and Huala, 1999), which are capable of detecting a wide spectrum of light wavelengths ranging from UV to far-red light.

Physiological and photochemical studies in recent decades have shown that presumed phytochrome-mediated responses in plants can be classified into three different response modes: red (R)/far-red (FR) reversible responses designated the low fluence response (LFR) and two other responses named the very low fluence response (VLFR) and the high irradiance response (HIR) according to their energy requirements (Mohr, 1962; Briggs et al., 1984). In a typical LFR, plants respond to 1 to 1000 μmol m$^{-2}$ of R light, whereas the VLFR requires only 0.1 to 1 μmol m$^{-2}$ of R light. The HIR requires prolonged exposure to light of relatively high photon flux and shows fluence rate dependence. Action spectra for VLFR, LFR (Shinomura et al., 1996), and HIR (Shinomura et al., 2000) using phytochrome-deficient mutants of Arabidopsis showed that phyA is the principal photoreceptor involved in the VLFRs, such as induction of seed germination (Shinomura et al., 1996) and CAB gene expression (Harnazato et al., 1997), and the FR light–induced HIRs, including inhibition of hypocotyl elongation (Quail et al., 1995; Shinomura et al., 2000). On the other hand, phyB controls LFR in a R/FR reversible mode.

Phytochromes in higher plants are encoded by a small gene family that is composed of five members (PHYA to PHYE) in Arabidopsis (Sharrock and Quail, 1989; Clack et al., 1994). Within the PHY gene family, phytochrome A (phyA) has been studied most extensively for its molecular properties and physiological function (Furuya, 1993; Quail et al., 1995) to identify its specific role(s) in a number of phytochrome-mediated events leading to plant growth and development. The mode of photoperception by phyA seems to be common among higher plants, but the function of phyA in vivo could be somewhat distinctive in different plant species at different developmental stages. For example, pea phyA mutants grown under long day photoperiods show a highly pleiotropic phenotype in mature plants, including short internodes, thickened stems, delayed flowering and senescence, longer peduncles, and higher seed yield (Weller et al., 1997), whereas the Arabidopsis phyA mutants...
grown under white light are morphologically indistinguishable from wild-type plants (Nagatani et al., 1993; Whitelam et al., 1993).

Although phytochromes are the most extensively characterized photoreceptors in plants, their diverse functions in the regulation of plant development have been characterized mainly in dicots, and little information in this regard is available in monocots. Phytochrome genes were isolated in monocots long ago (oat PHYA, Hershey et al., 1985; maize PHYA, Christensen and Quail, 1989; rice PHYA, Kay et al., 1989; rice PHYB, Dehesh et al., 1991), but the in vivo functions have not been elucidated, mainly due to the unavailability of phytochrome mutants. To date, the only phytochrome mutant found in monocots was a phyB mutant of sorghum that was originally identified as a ma3R early-flowering allele (Childs et al., 1997). Very recently, a phytochrome chromophore-deficient mutant was identified in rice (Izawa et al., 2000).

Rice has served as a model monocot plant system in which to explore complex genetic and physiological phenomena because a wealth of information is available on its genomic structure and functionality (Shimamoto, 1995). The task of designing and executing complex genetic studies in rice has been further facilitated by the availability of discrete research materials and tools, such as the high density genetic linkage map and thousands of expressed sequence tag clones made available by the Rice Genome Research Program (Yamamoto and Sasaki, 1997). A recent addition to these tools is the development of a gene knockout system using a rice retrotransposon named Tos17 (Hirochika et al., 1996; Hirochika, 1997, 1999), which makes it possible to isolate mutants for genes of interest. In this system, the active Tos17 can be used to easily generate a large number of Tos17-tagged mutant lines, and the mutants of a specific gene can be identified from the large mutant population (mutant panels) by polymerase chain reaction (PCR) using Tos17- and gene-specific primers.

The generation and characterization of phytochrome mutants of rice can broaden the existing view of phytochrome function in plants, especially in terms of the differences in phytochrome functionality in monocots and dicots. In this article, we report the isolation and characterization of a phyA mutant from a monocot (rice) plant. Because the mutant phenotype of rice deficient in phyA was unknown, we adapted the reverse genetics strategy to discover the rice phyA mutant. Physiological analyses of the phyA mutants at the young seedling stage revealed that phyA is responsible for the inhibition of coleoptile elongation that is produced by irradiation with a pulse of very low fluence R (VLF-R) light. Moreover, phyA also is involved in the inhibition of mesocotyl elongation and the induction of the gravitropic response in crown roots under continuous FR light. In contrast, phyA deficiency did not impart any noticeable morphological changes in plants grown under natural light conditions. The function of phyA in rice appears to be unique and restricted to the deetiolation process.

RESULTS

Isolation of Rice phyA Mutants from Mutant Panels

We screened a large population of Tos17 insertional mutants of rice by PCR to isolate rice phyA mutants. Three mu-

Figure 1. The Structure of the PHYA Gene and Insertion Sites of Tos17 in the PHYA Gene of Three Mutant Lines.

The structure of the PHYA gene is derived from sequence information available in the database (GenBank accession number X14172). The exon and intron regions of PHYA are presented as closed and open bars, respectively. Insertion sites of Tos17 sequences in the PHYA gene of three mutant lines are indicated by vertical bars labeled with the names of mutant lines and the range of nucleotides at the sites of insertion. The integrated Tos17 sequences are shown by shaded bars with insertion orientation. The locations of primers designed for PCR screening are indicated by arrows. PstI sites used for DNA gel blot analysis also are indicated.
insertion of Tos17 adds one more PstI site. As expected, we observed three banding patterns in the progeny of the osphyA-1 line: (1) homozygous for the insertion (Figure 2, lanes 4 and 5); (2) heterozygous (Figure 2, lane 3); and (3) wild type (Figure 2, lanes 1, 2, and 6). Because the number of plants showing these genotypes follows the Mendelian ratio of 1:2:1, it was demonstrated that the mutant alleles were stably transmitted to the next generation according to Mendel’s first law. We also confirmed the insertion of Tos17 into the PHYA gene and the normal inheritance of the mutant alleles in two other mutant lines, osphyA-2 and osphyA-3 (data not shown).

**Immunoochemical Analysis of the PHYA Apoprotein**

The level of PHYA apoprotein in the insertional mutants was determined by immunoblotting to examine if the mutant alleles of PHYA were null mutations. Figure 3 shows that the PHYA apoprotein was undetectable in crude protein extracts from etiolated phyA mutant progeny (osphyA-1-2 and osphyA-2-10), whereas progeny with wild-type PHYA alleles (osphyA-1-4 and osphyA-2-9) had similar levels of PHYA as the wild-type plants (cvs Akitakomachi and Nipponbare). Thus, the PHYA gene appeared to be completely knocked out in the osphyA-1 and osphyA-2 mutant lines.

**Effect of Continuous FR Light on Growth Responses in phyA Mutants**

We examined the effect of continuous irradiation with R and FR light on the growth responses of young wild-type and osphyA-1 mutant seedlings. The results are presented as original photographs of seedlings (Figure 4) and as measurements of mean lengths of coleoptile and mesocotyl
The coleoptiles and mesocotyls of the dark-grown seedlings reached their maximum lengths 6 days after the induction of germination without any additional changes for up to 19 days (data not shown).

Both the wild-type and osphyA-1 seedlings exhibited a typical elongation of coleoptiles and mesocotyls when grown in constant darkness (Figures 4 and 5D). The different appearance of the two dark-grown osphyA-1 seedlings in Figure 4 is due to a difference in the timing of germination. We did not observe significant differences in growth rate between wild type and phyA mutants. A commonly observed inhibition of coleoptile and mesocotyl elongation also was apparent when the seedlings of wild type and osphyA-1 mutants were exposed to continuous R light for 11 days. In this experiment, exposure to continuous R light reduced the growth of coleoptiles to one-sixth of that in dark-grown seedlings in both wild-type and osphyA-1 seedlings (Figures 4 and 5A, cR). Similarly, growth of the mesocotyl was inhibited completely under continuous R light in both lines (Figures 4 and 5B, cR). In contrast, growth responses of the osphyA-1 seedlings to continuous FR light were completely different from those of the wild type. When the seedlings were exposed to FR light for 11 days, the osphyA-1 coleoptiles and mesocotyls elongated like those of dark-grown seedlings (Figures 4B and 5, cFR), whereas wild-type coleoptiles were short (Figures 4A and 5A, cFR) and wild-type mesocotyls were undetectable (Figures 4A and 5B, cFR).

To determine the irradiation period with FR light needed for the detection of such growth inhibition, exposure times to FR light were gradually shortened until growth inhibition was no longer observed. Two-day-old etiolated seedlings, which are most sensitive to FR light for the inhibition of mesocotyl elongation, were exposed for various durations of continuous FR light from 1 hr to 5 days. After the irradiation, they were kept in darkness until the measurement of mesocotyl lengths in the 7th day. Their mesocotyl lengths were compared with those of seedlings grown in constant darkness as a control (Figure 5C). When the wild-type seedlings were exposed to continuous FR light for 3 hr or shorter, their mesocotyls were as long as those of seedlings grown in the dark (Figure 5C). However, when they were exposed for longer than 6 hr, their mesocotyl lengths became shorter as the duration of light exposure increased (Figure 5C). In contrast, the mesocotyl elongation of osphyA-1 mutants was not affected even by a 5-day exposure to continuous FR (Figure 5C). These results indicate that the inhibition of mesocotyl elongation is detectable in wild-type seedlings when exposed to a prolonged FR light irradiation (more than 6 hr). Essentially similar responses to continuous R and FR light were observed in another phyA mutant, osphyA-2 (data not shown). These results indicate that etiolated seedlings of the phyA mutants are insensitive to FR light for the modulation of coleoptile and mesocotyl length.

Effect of Light Pulse Irradiation on Coleoptile Growth

We examined the effect of R and FR light pulse irradiation with both low fluence (LF) and very low fluence (VLF) on the coleoptile growth of wild-type and osphyA-1 seedlings. Etiolated seedlings with coleoptiles longer than 10 mm were exposed to a pulse of LF-R, FR, or VLF-R light and were kept in the dark. Their coleoptile lengths were measured 2 days after the irradiation. Exposure to a pulse of LF-R light (~1 mmol m⁻²) completely inhibited the growth of coleoptiles in both the wild type and osphyA-1 (Figure 6, LF-R). Subsequent exposure to a pulse of FR light (~20 mmol m⁻²) partly reversed the effect of LF-R light exposure in the wild type (Figure 6, LF-R/FR). This LF-R/FR reversible response was observed much more clearly in osphyA-1 (Figure 6, LF-R, osphyA-1).

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Mutants Grown under Different Light Conditions.

Light (found that a pulse of either FR light (20 mmol m$^{-2}$) or VLF-R light (~1 μmol m$^{-2}$) was able to inhibit growth of the coleoptile in the wild type but not in osphyA-1 (Figure 6, VLF-R and FR). These results indicate that the inhibition of coleoptile elongation by a pulse irradiation of VLF-R or FR light in etiolated rice seedlings is mediated primarily by phyA.

Next, we examined the effect of VLF-R and FR light and found that a pulse of either FR light (20 mmol m$^{-2}$) or VLF-R light (~1 μmol m$^{-2}$) was able to inhibit growth of the coleoptile in the wild type but not in osphyA-1 (Figure 6, VLF-R and FR). These results indicate that the inhibition of coleoptile elongation by a pulse irradiation of VLF-R or FR light in etiolated rice seedlings is mediated primarily by phyA.

Root Gravitropic Response

We found another phenotypic difference in the root growth between wild-type and osphyA-1 seedlings. Growth orientations of crown roots under different light conditions are presented in Figure 7. In the dark, the crown roots of both wild-type and osphyA-1 seedlings grew mostly horizontally (Figures 7B and 7D). In other words, the crown roots of dark-grown rice seedlings are plagiotropic (growing parallel or nearly parallel to the surface). Under continuous R light, the gravitropic response of crown roots was induced and the roots grew with a peak frequency distribution at $+50$ to $+80^\circ$ in both wild-type and osphyA-1 seedlings (Figure 7B, cR). Wild-type seedlings grown in continuous FR light showed a similar geotropic response as seedlings grown in continuous R light, although the peak of the distribution in continuous FR light shifted slightly toward horizontal (Figure 7B, cFR). Crown roots of osphyA-1 were insensitive to continuous FR light, because their orientation was similar to that observed in the dark-grown seedlings (Figure 7B, cFR). These results indicate that the gravitropic response of rice crown roots is modulated by continuous irradiation with both R and FR light and that the phyA mutation caused the loss of response to continuous FR light.

We examined crown roots to find the duration of prolonged irradiation with FR light needed for the induction of the gravitropic response. Five crown roots emerge sequentially from the nodal portion of the rice coleoptile (Hoshikawa, 1989). We examined only the first of these five to emerge (Figure 7A, white arrowheads). Four-day-old etiolated seedlings, when the length of the first crown roots was $\sim 2 \pm 2$ mm (most sensitive stage to FR light for detection of this response), were exposed to various durations of continuous FR light from 15 min to 48 hr. They were grown in the dark until growth measurement at the 7-day-old stage. The growth angles of the first crown roots were measured and compared with those of seedlings grown in the dark or continuous FR light as a control (Figure 7C). When the wild-type seedlings were exposed to continuous FR light for 1 hr or less, the growth orientation of the crown root was equivalent to that of seedlings grown in the dark (Figure 7C). In one ~24-hr treatment, longer exposure times produced a larger growth angle in the first crown roots in wild-type seedlings (Figure 7C). The FR light irradiation for 48 hr caused the equivalent effect on this response obtained by continuous FR light irradiation (Figure 7C). In contrast, osphyA-1 mutants treated with various durations of continuous FR light showed growth orientation angles as large as those of seedlings grown in the dark (Figure 7C). Irradiation with VLF-R light (~1 μmol m$^{-2}$) did not induce the gravitropic response of the first growing crown root in wild-type seedlings (data not shown). Essentially, similar effects of continuous FR light and VLF-R light on the second growing crown roots were observed (data not shown). These results indicate that gravitropic growth orientation was induced only when the wild-type seedlings were exposed to a prolonged FR light irradiation (more than 3 hr).
The seminal roots, in contrast to crown roots, grew at an angle between $170^\circ$ and $190^\circ$ below horizontal, irrespective of the presence or absence of light in both wild-type and phyA mutant plants. The mean days-to-heading value for wild-type plants ($n = 21$) was $112.7 \pm 1.2$ days and that for osphyA-2-10 plants ($n = 70$) was $112.5 \pm 2.5$ days. We also raised the wild-type and mutant plants under short day (10.5 hr of light/13.5 hr of dark) conditions, but still no significant difference was observed for days to heading in either genotype (wild-type, $50.0 \pm 1.5$ days [$n = 32$]; osphyA-2-9 with wild-type allele, $51.7 \pm 1.4$ days [$n = 13$]; osphyA-2-10 with mutant allele, $51.6 \pm 1.7$ days [$n = 14$]). Therefore, rice phyA mutants were morphologically indistinguishable from wild-type plants when grown under natural light conditions.

Expression Patterns of Light-Regulated Genes

We determined the effect of R and FR irradiation on the abundance of transcripts for CAB and RBCS genes in the wild type and phyA mutants. Mutant and wild-type plants were grown in complete darkness for 7 days and exposed to continuous R or FR light. Control seedlings were kept in constant darkness during the experiment. The seedlings were harvested 0, 4, 12, and 24 hr after the irradiation and the accumulation of CAB and RBCS transcripts was investigated by RNA gel blot analysis. As shown in Figure 8A, RBCS transcripts were present at a low level even in the dark, and the amount of these transcripts was increased equally by both R and FR light in wild-type plants. In phyA mutant plants, R light exerted the same effect as in wild-type plants, as expected. Unexpectedly, however, RBCS gene expression was induced in phyA mutants by continuous FR light to almost the same level as in wild-type plants. In the case of CAB gene expression (Figure 8B), we were not able to detect any signal in the dark (column D or row 0 hr) samples. Both R and FR light induced an accumulation of CAB transcripts in wild-type plants, which showed a fluctuation due to circadian rhythms. In phyA mutant plants, R light exerted the same CAB gene expression pattern as in wild-type plants. FR light treatment also resulted in a similar fluctuation of CAB gene expression, although this expression level was much lower than that of wild-type plants. These results show that phyA seems to play a minor role in the induction of RBCS gene expression by FR light, whereas for the induction of CAB gene expression by FR light, phyA clearly play a more prominent role, although other photoreceptor(s) must also be involved.

DISCUSSION

Isolation of Rice phyA Mutants from Mutant Panels

In this article, we report the isolation and characterization of phyA mutants in a monocot (rice) plant. Identification and
characterization of mutants in living organisms is indispensable to efforts to understand and elucidate the function of particular genes. Mutants for phytochrome genes have been isolated in Arabidopsis and several other dicot species (Koornneef and Kendrick, 1994; Fankhauser and Chory, 1997). We used the gene knockout system (Hirochika, 1997, 1999) to isolate the rice phyA mutants, because isolation of mutants in rice by phenotypic selection was not as easy as in Arabidopsis.

Site-directed manipulation of chromosomal genes is a powerful tool with which to investigate the in situ function of isolated genes. A gene knockout system has been established in mice to produce phenotypes resulting from the elimination of functional genes or the loss of gene function. However, in plants, gene targeting has not become widely used because of the low frequency of homologous recombination (Thykjaer et al., 1997; Gallego et al., 1999; Jelesko et al., 1999). The transposon-tagging system provides an alternative method in plants to knock out specific genes. In this system, instead of targeting a specific gene, large populations

Figure 7. Effect of Light on the Growth Orientation of Crown Roots of Wild Type (WT) and phyA Mutants.

(A) Representative photographs of crown roots of wild-type (Akitakomachi) and osphyA-1 seedlings grown under continuous FR light for 11 days. White arrowheads indicate first growing crown roots. Bars = 0.5 cm.

(B) Histograms of growth angles of crown roots. Wild-type (Akitakomachi) and osphyA-1 seedlings were maintained in complete darkness (D) or were grown under continuous R light (cR) or continuous FR light (cFR) for 11 days. Angles of crown roots were measured from the horizontal axis and divided into 10° intervals. The numbers of crown roots of wild-type and osphyA-1 seedlings are indicated by light blue and magenta bars, respectively.

(C) Effects of continuous FR light on the gravitropic growth orientation of the first growing crown roots (indicated by white arrowheads in [A]). Four-day-old etiolated seedlings of wild type (Akitakomachi; light blue bars) and osphyA-1 mutants (magenta bars) were irradiated with continuous FR light for intervals ranging from 15 min to 48 hr and kept in darkness until the 7th day. The growth angles of the first-grown-crown roots were measured. As controls, those of the seedlings grown in the dark or under the continuous FR light are shown. Error bars represent sE; n = 20.
of mutants (mutant panels) are generated by random insertion of retrotransposons and the mutants for a gene of interest are isolated by PCR-based screening of mutant panels. We used this system to isolate several independent lines in which the PHYA gene was knocked out.

In each of the phyA mutant lines, an exon of the PHYA gene was interrupted by the insertion of Tos17 sequence (Figure 1) and an in-frame stop codon was produced just downstream of the insertion site. These sequences have the potential to produce truncated PHYA molecules. However, the aberrant transcripts corresponding to the truncated PHYA molecules seem not to be translated, because immunodetectable bands were absent from two mutant lines (osphyA-1-2 and osphyA-2-10; Figure 3). The transcript level of the PHYA gene also was determined in the etiolated seedlings by RNA gel blot analysis. An expected, the transcript length of PHYA (4 kb) was not detected in either osphyA-1-2 or osphyA-2-10. Therefore, we categorized these mutant lines as null mutants for the phyA function.

Each mutant line in the mutant panels that we used has 5 to 10 copies of transposed Tos17. Thus, it is important to eliminate the possible effects of the insertion of Tos17s into genes other than PHYA. Fortunately, we were able to isolate three independent mutant lines (osphyA-1, osphyA-2, and osphyA-3) and found the same phenotypes from the two mutant lines we checked (osphyA-1 and osphyA-2), which proved that the phenotypes observed in the present study were caused by the loss of function of PHYA.

Role of phyA in the Photomorphogenesis of Rice Seedlings

Screening of the mutant panels resulted in the isolation of three allelic phyA mutants that show a dramatically reduced morphological response to continuous FR light, including inhibition of coleoptile and mesocotyl elongation and of the gravitropic response of crown roots (Figures 4, 5, and 7). Together with the sequence analysis (Figure 1) and immunochemical data (Figure 2), these results indicate that the altered photomorphogenesis in the phyA mutants results from a deficiency in functional phyA and that phyA is the predominant phytochrome in the dark-grown seedlings that mediates deetiolation responses induced by continuous FR light in rice. On the other hand, when grown under continuous R light (Figures 4, 5, and 7) or white light (data not shown), the phenotypic properties of phyA seedlings were essentially the same as those of the wild type, suggesting that phyA is mostly dispensable for the photomorphogenesis of rice seedlings under these conditions.

A pulse irradiation of LF-R light is known to inhibit the elongation of coleoptiles of rice seedlings when the coleoptile is longer than 10 mm (Pjon and Furuya, 1967). This response was partially reversed by subsequent irradiation with a pulse of FR light, indicating that it was the R/FR reversible response (Pjon and Furuya, 1967). In the present study, we repeated the same experiment for the phyA mutants and found that they showed significantly clearer reversibility of R/FR response compared with the wild type (Figure 6, LF-R, LF-R/FR, and LF-R/FR/LFR). We think that in the wild type, the FR pulse–induced reverse response mediated mainly by phyB was almost canceled by phyA-mediated growth inhibition induced by the FR pulse. The results presented here clearly show that the coleoptile elongation in wild-type plants is inhibited by a FR light pulse and are consistent with the results using a single short exposure (Pjon and Furuya, 1967) or hourly pulses of FR light exposure (Casal et al., 1996). In addition, we have demonstrated that a pulse of VLF-R or FR light that inhibited the coleoptile elongation in wild-type seedlings was not effective for the inhibition of coleoptile elongation in phyA mutants (Figure 6, Figure 8.)
crown roots, which come out from higher nodes, start de-

doping and tend to develop horizontally. Because the

itropic response. In rice, crown roots emerge from nodal

maize, the primary roots show the light-induced gravitro-

verse the R light induction (Feldman and Briggs, 1987). However,

these observations suggested the involvement of multiple pho-

tropein species mediated by VLFR and FR light, and photo-

trope receptors. Arabidopsis was the only plant species in which

expression for genes involved in photosynthesis (Kuno et al.,
2000). In particular, \textit{CAB} and \textit{RBCS} genes are well-characterized nuclear genes whose expression is regulated by phytochromes at the transcriptional level. The loss of phyA function in the \textit{phyA} mutants should alter the expression pattern of \textit{CAB} and \textit{RBCS} genes. In Arabidopsis, it was reported that irradiation with both VLFR and FR light could induce the expression of the \textit{CAB} gene in \textit{phyB} mutants, whereas in the \textit{phyA} mutants, \textit{CAB} gene expression could be induced by LF-R light but not by FR light (Hamazato et al., 1997). These results suggest that phyA is the sole photoreceptor in Arabidopsis that can induce \textit{CAB} gene expression upon irradiation with FR light in dark-grown seedlings.

In rice \textit{phyA} mutants, however, FR light induced \textit{RBCS} and \textit{CAB} gene expression in dark-grown seedlings at different levels. The induction of \textit{RBCS} gene expression was almost to the same level as in wild-type plants (Figure 8A), whereas the induction level of \textit{CAB} gene expression was much less in \textit{phyA} mutants than in wild-type plants (Figure 8B). These results show that phyA contributes to the accumulation of \textit{RBCS} and \textit{CAB} transcripts differently under FR light and also suggest that photoreceptor(s) other than phyA can mediate the expression of these genes in response to irradiation with FR light in etiolated rice seedlings. Only three phytochrome genes (\textit{PHYA}, \textit{PHYB}, and \textit{PHYC}) have been reported to date in rice (Tahir et al., 1998), and phyB is assumed to be a sensor for classic R/FR photoreversible responses (Shinomura et al., 1996). Therefore, phyC could be a candidate photoreceptor for such photoreponse. In Arabidopsis, phyC is thought to be a type II phytochrome based on its photostable nature and its small amount in both etiolated and green plants, but its photoregulatory roles have not been fully elucidated because monogenic mutants of phyC have yet to be isolated (Quail, 1998). Thus, we cannot exclude the possibility that phyC mediates the \textit{CAB} gene expression induced by FR light. Regardless of the facts to be established in the future, the present study demonstrates that a photoreceptor(s) other than phyA perceives FR light for the induction of \textit{CAB} gene in the etiolated seedlings.

Modes of phyA Action in Rice

The present study has shown that the photoinhibition of coleoptile elongation in rice induced by a pulse of light irradiation is mediated by both phyA in VLFR mode and probably phyB in LFR mode. In contrast, the photoinhibition of mesocotyl elongation and the light-dependent gravitropic response of crown roots are HIRs and are mediated by phyA under continuous FR light and by some other phytochrome(s) under continuous R light.

The results presented here clearly show that rice phyA controls photomorphogenesis in two distinct modes of photoperception—VLFR and HIR—and are consistent with the results obtained for \textit{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., 1995). In Arabidopsis, phyA is known to control development and growth (Chory et al., 1996) in several different modes of action, including seed germination in VLFR mode (Shinomura et al., 1996) and inhibition of hypocotyl elongation in FR light–induced HIR mode (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). Therefore, phyA seems to play a similar role in the deetiolation of monocot and dicot plants, in spite of the fact that FR light induces the inhibition of coleoptile elongation in monocots (i.e., rice) and hypocotyl elongation in dicots (i.e., Arabidopsis). Although elongation inhibition of both coleoptiles and hypocotyls seems to result from the inhibition of cell elongation, the modes of photoperception by phyA in those responses are quite different, the former being via VLFR and the latter being via HIR (Shinomura et al., 2000).

This difference raises the question of whether the two modes of responses via phyA—VLFR and HIR—are completely different or share some overlapping pathways. Recent findings in phytochrome biochemistry, including nuclear localization and kinase activity, might lead us to a plausible explanation for these physiological responses (Reed, 1999; Neff et al., 2000). Important work in this regard includes the following: (1) intracellular localization studies of phyA (Kircher et al., 1999; Hisada et al., 2000); (2) the demonstration of light-dependent kinase activity of oat phyAs (Wong et al., 1989; Biermann et al., 1994; Yeh and Lagarias, 1998); and (3) the identification of a protein that can be phosphorylated by oat phyA in a light-regulated manner (Fankhauser et al., 1999). On the basis of such information, it can be hypothesized that phyA signaling for photomorphogenesis responses may depend on combinations of the variables, including nuclear versus cytoplasmic location, phosphorylated versus unphosphorylated, and dark-synthesized Pr, photoconverted Pfr, and photocycled Pr. It remains to be determined in the near future which proteins interact with each species of phyA described above and how these various control mechanisms interact with each other.

Perspective

In rice, a minimum of three phytochrome genes (\textit{PHYA}, \textit{PHYB}, and \textit{PHYC}) are expressed (Tahir et al., 1998). Recent evidence in Arabidopsis indicates that individual members of the phytochrome family show different manners of photoperception (Shinomura et al., 1996) and have specialized functions (Reed et al., 1994; Quail, 1998; Whitelam et al., 1998). To characterize the functions of all known phytochromes in rice, we have also been attempting to isolate \textit{phyB} and \textit{phyC} mutants from the mutant panels of rice, but no candidates have been obtained to date. On the other hand, we recently identified three additional mutant alleles of the \textit{PHYA} gene from the mutant panels. Previously, Hirochika et al. (1996) analyzed the border sequences of eight random
Table 1. Nucleotide Sequences of PHYA- and Tos17-Specific Primers

<table>
<thead>
<tr>
<th>PHYA-Specific Primer Name</th>
<th>PHYA-Specific Primer Sequence</th>
<th>Primer Position</th>
<th>Tos17-Specific Primer Name</th>
<th>Tos17-Specific Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF</td>
<td>GAAGCATGGAGGTGCTATGC</td>
<td>5278–5297</td>
<td>LTR1</td>
<td>TTGGATCTTGATCTTGATATAC</td>
</tr>
<tr>
<td>AF1</td>
<td>TATGCATACGGTGCTAGAGGCC</td>
<td>5293–5313</td>
<td>LTR2</td>
<td>GCTAATACATGTGGAGTGGCAA</td>
</tr>
<tr>
<td>BF</td>
<td>CCAACATATGAGGTACCGTGG</td>
<td>5944–5963</td>
<td>LTR3</td>
<td>CTGGACATGGGACACATACAGT</td>
</tr>
<tr>
<td>CF</td>
<td>CTTCATCATCGAGTTCTAGC</td>
<td>4327–4347</td>
<td>LTR4</td>
<td>CCAATGGACTGGACATCCGATGG</td>
</tr>
<tr>
<td>BF1</td>
<td>ACAGCTATGCTTGGTCTGCG</td>
<td>6038–6057</td>
<td>LTR5</td>
<td>CCAATGGACTGGGAGACATCCGATGG</td>
</tr>
<tr>
<td>CF1</td>
<td>ATCTTGTTCAGCAGGAGTGG</td>
<td>4558–4577</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>AGGATGAAAGGTGACATGCC</td>
<td>8583–8564</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER1</td>
<td>GACATCGCCATTGATGAGGCC</td>
<td>8545–8527</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aAF1, BF1, CF1, and ER1 are the nested PCR primers of AF, BF, CF, and ER, respectively.
*bPosition of the first and last nucleotides in phy18 (PHYA) sequence (GenBank accession number X14172).
*cLTR2 and LTR5 are the nested PCR primers of LTR1 and LTR4, respectively.

Tos17 insertion sites and found one of them to be a PHYA gene. Therefore, the chromosomal region where PHYA resides appears to be a focus for the transposition of Tos17. In fact, the findings of Hirochika’s group inspired us to attempt the isolation of Tos17 insertional mutants of phytochrome genes in rice. We have screened 11,600 mutant lines so far, but our failure to obtain the phyB and phyC mutants indicates that Tos17-induced mutations must be saturated to make this system applicable to any gene of interest. We would need to produce a large number of mutagenized lines to achieve this saturation level, and further screening of the upcoming panels is expected to reveal the phyB and phyC mutants.

METHODS

Isolation of phyA Mutants from Mutant Panels

Large populations of rice (Oryza sativa) mutants generated by means of Tos17-mediated mutagenesis were made available by the Laboratory of Gene Function at the National Institute of Agrobiological Resources (Tsukuba, Japan). Details of mutagenesis with Tos17 have been described (Hirochika, 1999). To identify phyA mutants from a large mutant population, the mutant lines were divided into several groups, and within each group the lines were aligned as two- or three-dimensional matrices. A particular group of mutant lines was termed a “mutant panel.” For example, a mutant panel of 625 lines consisted of a matrix in which all of the mutant lines were aligned in 25 rows and 25 columns. Plant samples were collected and mixed to make a pool for DNA extraction from all of the individual mutants in a particular row or column. The pooled DNA was subjected to polymerase chain reaction (PCR) analysis so that a maximum of 50 (25 × 25) PCR reactions were enough to survey 625 mutant lines. A mutant line with the insertion of Tos17 in a specific gene could be identified by using primers specific to the gene of interest and Tos17. If a desired mutant exists in the panel, DNA pools from one row and one column must give amplified DNA fragments of the same size, and the mutant line can be addressed on the matrix by the row/column number.

Screening for the phyA Mutant by PCR Amplification

We screened three mutant panels (NA0, NB0, and NC0) for the isolation of phyA mutants. NA0 was a two-dimensional (23 × 24) mutant panel, whereas NB0 and NC0 were three-dimensional (8 × 10 × 12) panels. Table 1 lists the details of two Tos17-specific primers (LTR1 and LTR4) and four PHYA-specific primers (AF, BF, CF, and ER) that were used in all possible combinations for PCR amplification of 300 ng of genomic DNA from each pooled DNA sample. To eliminate nonspecific amplifications, we designed additional PHYA-specific (AF1, BF1, CF1, and ER1) and Tos17-specific (LTR2 and LTR5) primers just upstream of each primer for nested PCR (Table 1). The amplified DNA fragments were cloned and sequenced to confirm the insertion of Tos17.

Genomic DNA Isolation and DNA Gel Blot Hybridization

Genomic DNA was isolated from young leaves using the cetyl-trimethyl-ammonium bromide method (Murray and Thompson, 1980). The genotype analysis was performed using 2 μg of genomic DNA digested with PstI, fractionated on an 0.8% agarose gel, and transferred to Hybond N+ membranes (Amersham Pharmacia Biotech). A full-length clone of PHYA cDNA was random labeled and used as a probe for the genotype analysis.

Immunochemical Analysis

Crude protein extracts were prepared from 7-day-old etiolated seedlings of wild type and phyA mutants for the immunochemical detection of PHYA. Approximately 0.5 g of seedlings was homogenized in the presence of 1 mL of phytochrome extraction buffer (100 mM Tris-HCl, pH 8.3, 5 mM EDTA, 0.2% 2-mercaptoethanol, and 100 mM phenylmethylsulfonyl fluoride). The supernatant was recovered after centrifugation at 10,000g for 15 min twice, and 0.66 volume of saturated ammonium sulfate was added. The resultant precipitate was dissolved in 100 μL of phytochrome extraction buffer, and the protein concentration was determined. Fifty micrograms of each protein extract was separated by SDS-PAGE (10% acrylamide gel) and electrophoretically transferred to a nitrocellulose membrane. The monoclonal antibody used in this study was the anti-rye PHYA antibody (mAR07) from the
monoclonal antibody stock at the Hitachi Advanced Research Laboratory (Hatoyma, Japan). Immunochemical detection was performed using an immunoblot kit (alkaline phosphatase system; Bio-Rad).

**Plant-Growth Measurement and Light Treatment**

For measurements of coleoptile and mesocotyl length, dehusked rice seed were surface sterilized and placed on 0.6% agar in glass tubes. The glass tubes were kept in a light-tight box at 4°C for 3 days. Seed were placed on the agar so that embryos had an upward orientation. The seed in the glass tubes were transferred subsequently to 25°C in the dark for 24 hr before the light treatment. In the experiment with continuous light irradiation, seedlings were exposed to continuous red (R) light (photon fluence of 50 μmol m⁻² sec⁻¹) or continuous far-red (FR) light (photon fluence of 90 μmol m⁻² sec⁻¹) for 11 days. For the experiments with different intervals of continuous FR light treatments on mesocotyl growth, 2-day-old etiolated seedlings were irradiated with continuous FR light for intervals ranging from 1 hr to 5 days and kept in darkness until the measurement on the 7th day. For irradiation with pulses of light, 4-day-old etiolated seedlings with coleoptile lengths of 13.2 ± 1.5 mm in the wild type and 10.9 ± 1.4 mm in osphyA-1 were exposed to a pulse of R light (17.8 μmol m⁻² sec⁻¹) for 56 sec for low fluence R [LF-R], 142 nmol m⁻² sec⁻¹ for 7 sec for very low fluence [VLF-R] and/or FR light (28.4 μmol m⁻² sec⁻¹ for 703 sec) and then kept in the dark for 2 days until the measurement. The lengths of coleoptiles and mesocotyls were measured using a digimatic caliper (CD-15C; Mitsutoyo, Tokyo, Japan). The R and FR light were obtained using broad-band filters according to Shinomura et al. (1994). For measurement of the growth orientation of root growth was measured using a protractor as the angle between the horizontal plane (0°) and the orientation of the proximal 10-mm part of crown roots of 10 to 30 mm in length (or of the seminal root of 20 to 80 mm in length). Orthogravitropic orientation was measured as a positive angle. All experiments were conducted three times with 30 to 50 plants per data point. To examine the effect of different intervals of continuous FR light treatments on gravitropic growth orientation of the first growing crown roots, 4-day-old etiolated seedlings were irradiated with continuous FR light for intervals ranging from 15 min to 48 hr and kept in darkness until the measurement on the 7th day.

**REFERENCES**


