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OVEREXPRESSION OF RICE PHYTOCHROME A IN *ARABIDOPSIS*: DIVERSE ROLE IN MULTIPLE PHYSIOLOGICAL RESPONSES

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Abstract

Phytochrome A predominantly mediates responses to prolonged far-red light such as the inhibition of hypocotyl elongation. It also acts in controlling responses to FR light pulses. To investigate to what extent rice phytochrome A complements the function of *Arabidopsis* phytochrome A, we examined hypocotyl elongation growth, seed germination, and greening in etiolated seedlings of *phyA-211* mutant plants, WT and two kinds of transgenic plants in which WT and *phyA-211* mutants overexpressed rice *PHYA* cDNA. We found that ectopic expression of rice phytochrome A could partially rescue the sensitivity of *phyA-211* mutant hypocotyls to prolonged far-red light and replace *Arabidopsis* phytochrome A in the promotion of seed germination and recovery of the ability to de-etiolate. Moreover, under end-of-day far-red light (EOD-FR), the exogenous rice phytochrome A was found to restore some aspects of growth in *phyA-211* mutants, such as hypocotyl elongation, petiole elongation and flowering time. The diverse role of rice phytochrome A in the physiological responses of *Arabidopsis* implies the substitution of phytochrome signaling between monocots and dicots.

Abbreviations: CAB, chlorophyll a/b binding protein; D, dark; EOD-FR, end-of-day far-red light; FR, far-red; HIR, high-irradiance response; LFR, low-fluence response; OsPHYA, rice phytochrome A; Pfr, far-red light-absorbing form of phytochrome; Pr, red light-absorbing form of phytochrome; R, red; R/FR, quantum ratio of red and far-red light; VLFR, very-low-fluence response; W, white; WT, wild type.

Introduction

Light determines the strategy of plant growth and development throughout the organism's entire life cycle. Plants have exquisite sensory systems that monitor the intensity, quality, direction, and duration of light. The various developmental responses of plants to light signals are collectively referred to as photomorphogenesis (Whitelam *et al*., 1998). In *Arabidopsis*, there are several distinct photoreceptors that sense light signals. The most extensively studied photoreceptors, which absorb mainly red (R) and far-red (FR) light, are phytochromes (Quail, 2002). The phytochrome family displays both unique and overlapping roles throughout the life cycle of plants, regulating a wide range of developmental processes from seed germination to the timing of reproductive development (Franklin & Quail, 2010).

Phytochrome exists in the form of homodimers, and each of monomer contains a covalently attached tetrapyrole (bilin) chromophore. These proteins regulate numerous photoresponses in plants and microorganisms through their ability to photointerconvert between a red-light-absorbing, ground state (Pr) and a far-red-light-absorbing, photoactivated state (Pfr) (Rockwell *et al*., 2006). Phytochromes are a collection of bilin-containing photoreceptors with a C-terminal regulatory domain and a conserved N-terminal photosensory domain that typically includes a histidine-kinase-related region and two PAS-domains (Rockwell *et al*., 2006).

All higher plants possess multiple discrete phytochromes, which are closely related and encoded by a small family of divergent genes. In *Arabidopsis thaliana*, five apophytochrome genes (*PHYA*-*PHYE*) have been isolated and characterized (Sharrock & Quail, 1989; Clack *et al*., 1994). Phytochrome A (PhyA) is light liable and has been shown to predominate in etiolated seedlings, while the other phytochromes (phyB-phyE) are less abundant and appear to be more light-stable (Kendrick & Kronenberg, 1994). Counterparts of *PHYA*, *PHYB*, and other *PHY* genes are not only present in higher plants but also in many prokaryotic species and fungi (Lamparter, 2004; Mathews, 2006).

PhyA mediates three distinct photobiological responses in plants: the very-low-fluence response (VLFR), which can be saturated by short pulses of very-low-fluence light; the high-irradiance response (HIR), which requires prolonged irradiation with higher fluences of far-red light; and, to some extent, the low-fluence response (LFR) (Kneissl *et al*., 2008). PhyA is the predominant form in etiolated tissues. It mediates germination, de-etiolation, and perception of day length under continuous FR light (Whitelam *et al*., 1993; van Tuinen *et al*., 1995; Shinomura *et al*., 2000; Takano *et al*., 2001). Due to rapid protein degradation and limited transcription in response to light, phyA has a reduced ability to antagonize shade avoidance responses (Sharrock & Clack, 2002). PhyA also contributes to seedling de-etiolation, leaf development, plant architecture, and root phototropism (Kiss *et al*., 2003; Tepperman *et al*., 2006; Franklin *et al*., 2007). Furthermore, phyA plays a minor role in the perception of continuous red light in *Arabidopsis* seedlings during de-etiolation and induction of the expression of chlorophyll a/b binding protein (CAB) genes (Reed *et al*., 1993).

Rice (*Oryza sativa* L.) serves as a model monocot. The wealth of information available on its genomic structure and function, makes it useful for exploration of many complicated genetic and physiological phenomena (Reed *et al*., 1993). It has been reported that rice has only three phytochrome genes: *OsPHYA*, *OsPHYB*, and *OsPHYC* (Dehesh *et al*., 1991; Tahir *et al*., 1998; Basu *et*

al., 2000). Both phyA and phyB mediate the low-fluence response, phyA and phyC are involved in the photoperception of continuous far red light in rice (Dehesh *et al*., 1991; Tahir *et al*., 1998). In rice, phytochromes were found to show synergistic, redundant, and antagonistic effects on the determination of flowering time determination in response to day length, and these effects differed from those seen in *Arabidopsis* (Takano *et al*., 2005). So far, ectopic expression of monocot phyA from oats and rice has been studied in heterologous systems (Stockhaus *et al*., 1992; Boylan *et al*., 1994; Emmler *et al*., 1995; Jordan *et al*., 1995; Jordan *et al*., 1997; Halliday *et al*., 1999; Casal *et al*., 2002; Kneissl *et al*., 2008). In these studies, overexpression of *PHYA* resulted in a hypersensitive phenotype under high FR irradiance and exhibited inhibited hypocotyl elongation under different light conditions, with one exception of the overexpression of *OsPHA* in tobacco (Emmler *et al*., 1995). Recently, it has been reported that the OsphyA can complement VLFR and LFR responses, such as inhibition of hypocotyl elongation under pulses of FR or continuous red light, induction of flowering, and leaf expansion (Kneissl *et al*., 2008). To better understand whether rice phyA can replace *Arabidopsis* phyA in different light responses, we studied the function of rice phyA in *Arabidopsis*. After a detailed function analysis of rice phyA in seed germination, hypocotyl elongation growth, greening of the etiolated seedlings, and the seedling responses to end-of-day far-red light (EOD-FR) treatments, we found that ectopic expression of *OsPHYA* partially rescued the sensitivity of *phyA-211* hypocotyls to prolonged far-red light and replaced the function of phyA in promoting seed germination and recovery of de-etiolating ability. We also observed a restoration of development growth in the *phyA-211* mutant, such as hypocotyl elongation, petiole elongation, and flowering time under EOD-FR. These observations suggest that rice phyA plays diverse role in several physiological responses in transgenic *Arabidopsis*.

Materials and Methods

Plant material and construction of transgenic plants: Wild type (WT) Arabidopsis (*Arabidopsis thaliana*), *phyA-211* mutant, and all transgenic lines used in this study were from the Columbia ecotype background (Reed *et al*., 1994). Full-length rice *PHYA* cDNA (Locus_ID: Os03g0719800) was cloned into cloning vector pSAT6-*EYFP*-C1 to generate pSAT6-*EYFP*-C1-*OsPHYA*. The expression cassette *2X35S::EYFP*-C1-*OsPHYA* was subcloned into the pRCS2-ocs-bar binary vector at the PI-Psp1 site. The constructed plasmid was transferred into WT *Arabidopsis* and the *phyA-211* mutant via the *Agrobacterium*-mediated floral dipping transformation method (Clough & Bent, 1998). Homozygous lines were isolated by selecting for basta resistance in the T_2 progeny. For phenotypic characterization, T_4 generations were used.

Growth conditions: Seeds were harvested from mature plants grown under natural photoperiods (16 W/8 D, photon irradiance, 100 μmol m^{-2} s^{-1} at 23 °C). For all phenotypical analyses, seeds were surface-sterilized for 2 minutes in 75% ethanol (v/v), followed by 5 minutes in 10% NaClO solution (w/v), washed five times in sterile distilled water, and plated on growth medium (MS medium, 1.5% sucrose (w/v), 0.8% agar (w/v) and pH 5.7). Then they were kept at 4 °C for 4 days in the dark to break dormancy (stratification) in order to germinate.

Light sources: R and FR light were generated by LED light sources with a 720–735 nm maximum (E-30LED, Percival, USA.). Light intensities were determined with a Radiometer Photometer (SKP 200, Hansateck, UK). White light was supplied by cool-white fluorescent lamps (TLD 36 W/ 54–765, Philips).

Protein extraction and immunoblotting: Surface-sterilized seeds were germinated on MS medium under continuous white light for 3 weeks. About 300 mg plant tissue was collected in the mortar and ground thoroughly with liquid nitrogen. Tissue powder was mixed with 200 μl extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40 (v/v) , 4 M urea, 1 mM PMSF) for denaturing protein. Then the solution was resuspended thoroughly, kept on ice for 10 minutes, boiled for 5 minutes, and clarified by centrifugation at 12,000 rpm for 10 minutes at 4 °C twice. The clear supernatant was aspirated for protein assay. Protein concentration was determined by BCA protein assay reagent using BSA as standard (Pierce). 2×SDS loading buffer was applied and samples were incubated at 100 °C for 5 minutes. Rabbit monoclonal anti-GFP D5.1 antibody (Cell Signaling Technology) and goat anti-rabbit IgG (Abcam) were used in immunoblotting. SDS-PAGE and immunoblotting were performed as previously reported (Kneissl *et al*., 2008).

Determination of hypocotyl length under continuous light conditions: After stratification, germination was induced by a 15-minute white light pulse (100 μmol m^{-2} s^{-1}). The seeds were then kept in the dark overnight at 23 °C. Seedlings were then treated with continuous FR light (2 μmol m^{-2} s^{-1}), continuous R light (28 μmol m^{-2} s^{-1}), continuous white light (100 μmol m^{-2} s^{-1}), or placed in darkness for 4 days, respectively. Hypocotyls were photographed with a Sony DSC-H50 camera and measured with DIGIMIZER 3.2.1.0 (http://www.digimizer.com).

Germination assays: All seeds were surface-sterilized and stored at 25 °C for two weeks and then placed on plates with moistened filter paper. Plates were immediately exposed to FR light (2 μmol m^{-2} s^{-1}) to inhibit phyB-dependent dark germination and then kept in darkness at 7 °C for 3 days, at 35 °C for 8 hours, and at 25 °C for 0.5 hours. The following treatments were performed in different assays: (1) A 3-minute pulse of FR light (0.04, 0.5, 2.0 μmol m^{-2} s^{-1}) was applied and then plates were kept in darkness at 23 °C for 4 days; (2) a R/FR irradiation ratio of 0.04 was provided for 7 days; (3) a 3-minute pulse of a R/FR ratio of 0.04 was administered at different frequencies and then plates were placed in darkness at 23 °C for 4 days (Botto *et al*., 1996). After these treatments, germination was scored using a microscope to assess radicle emergence. The germination rate was calculated.

Greening assays and determination of chlorophyll content: After stratification, seeds were incubated for 16 hours in continuous white light (100 μmol m^{-2} s^{-1}) and then kept in darkness for different lengths of time. Then the etiolated seedlings were transferred to natural photoperiods for 3 days and the proportion of de-etiolated seedlings was measured (Nagatani *et al*., 1993). The etiolated seedlings exposed to 4 days of darkness were irradiated with a 3-minute pulse of red light (28 μmol m^{-2} s^{-1}) and kept in darkness for 4 hours. They were then transferred to continuous white light for different lengths of time. Chlorophyll content was determined photometrically by measuring absorption at 663 and 645 nm (Yang, 2002). Chlorophyll content was calculated by the following formula and conversion unit is mg. g^{-1} FW.

Chlorophyll a (mg/l) = $12.7 \times A_{663} - 2.69 \times A_{645}$

Chlorophyll b (mg/l) = $22.9 \times A_{645} - 4.68 \times A_{663}$

Chlorophyll (mg/l) = Chlorophyll a + Chlorophyll b

Determination of responses to end-of-day far-red light treatments: After stratification, seeds were incubated in continuous white light (100 μmol m^{-2} s^{-1}) for 2 or 5 days. Two-day-old seedlings were treated with EOD-FR treatment with white light (100 μmol m^{-2} s^{-1}) for 9 hours, pulses of FR light (12 μmol m^{-2} s^{-1}) for 10 minutes, and darkness for 14 hours and 50 minutes. Then hypocotyls were photographed using a Sony DSC-H50 camera and measured with DIGIMIZER 3.2.1.0 (http://www.digimizer.com). Five-day-old seedlings were treated with EOD-FR and white light (100 μmol m^{-2} s^{-1}) for 9 hours, pulses of FR light (12 μmol m^{-2} s^{-1}) for 10 minutes, and darkness for 14 hours and 50 minutes to florescence. Then the amount of petiole elongation was measured with DIGIMIZER 3.2.1.0 (http://www.digimizer.com). The number of rosette leaves at inflorescence was used to represent flowering time.

Results

Molecular characterization of *Arabidopsis* lines ectopically expressing rice *PHYA*: To investigate to what extent rice phyA can replace *Arabidopsis* phyA, we examined hypocotyl elongation growth, seed germination, greening of etiolated seedlings, petiole elongation, and flowering time in *phyA-211* mutants and WT plants transformed with a pSAT6-*EYFP*-C1-*OsPHYA* fusion under the control of the CaMV 35S promoter. The protein levels in the transgenic plants were determined by immunoblot analysis using extracts from 3-week-old light-grown seedlings. Because of the constitutive CaMV 35S promoter, the protein levels were high in transgenic plants (Fig. 1). No detectable bands were observed in the WT or *phyA-211* seedlings, indicating that *OsPHYA* was successfully expressed in *Arabidopsis*. We selected two independent lines, *35S::EYFP-OsPHYA*/Col and *35S::EYFP-OsPHYA*/*phyA-211*, to analyze their responses under different light treatment conditions.

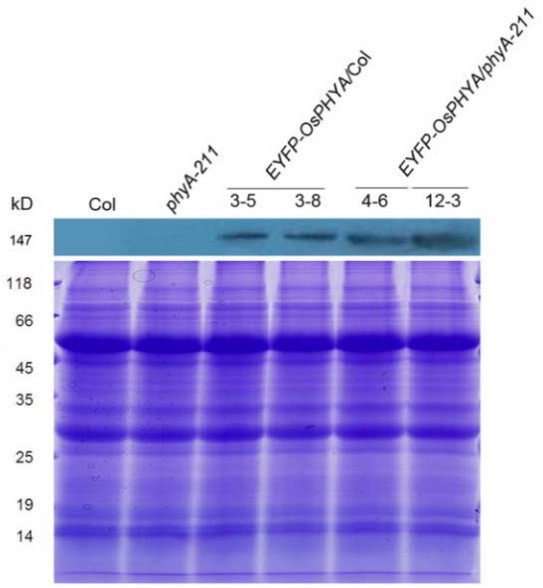

Fig. 1. Immunoblot analysis of rice phytochrome A levels in *Arabidopsis* lines expressing rice *PHYA*. Total protein extracts were prepared from 3-week-old light-grown seedlings. OsphyA was detected with specific antibody (upper panel). The lower panel shows a Coomassie-blue-stained SDS-polyacrylamide gel confirming equal amounts of total protein in the extract. Lane 1, Col; lane 2, *phyA-211* mutant; lanes 3 and 4, *35S::EYFP-OsPHYA*/Col; lanes 5 and 6, *35S::EYFP-OsPHYA*/*phyA-211*.

Hypocotyl elongation under R, FR, and white light: In photomorphogenesis, hypocotyl elongation was regarded as the standard test for light response because of its fluence dependency (Kneissl *et al*., 2008). For all genotypes, dark-grown seedlings exhibited extremely elongated hypocotyls of similar lengths (Fig. 2A and 2B). Hypocotyl elongation in *phyA-211* mutants under FR-light conditions was comparable to that seen in dark-grown seedlings. Under R and white light, hypocotyl elongation was found to be noticeably inhibited, probably because phyB, C, D, and E were still functional. Especially, phyB played a primary role. White-light-treated seedlings displayed identical inhibition of hypocotyl elongation. Red-light-treated seedlings showed a similar response pattern to that seen in white-light-treated seedlings, but hypocotyl length was shorter than that under white light treatment (Fig. 2B). These data suggest that compound light is more efficient than monochromatic light in the inhibition of hypocotyl elongation.

Under continuous FR light (HIR), expression of *OsPHYA* in *phyA-211* mutants reduced hypocotyl length slightly but not to WT levels. This suggests that rice phyA can not completely complement *Arabidopsis* phyA under FR light (Fig. 2B). However, a severe reduction in hypocotyl length was observed in *35S::EYFP-OsPHYA*/Col, suggesting that both endogenous phyA and exogenous phyA play a role in the inhibition of hypocotyl elongation. The function of rice phyA may be similar but is probably not identical to that of *Arabidopsis* phyA. In conclusion, under continuous FR HIR conditions, rice phyA was found capable of partially replacing *Arabidopsis* phyA.

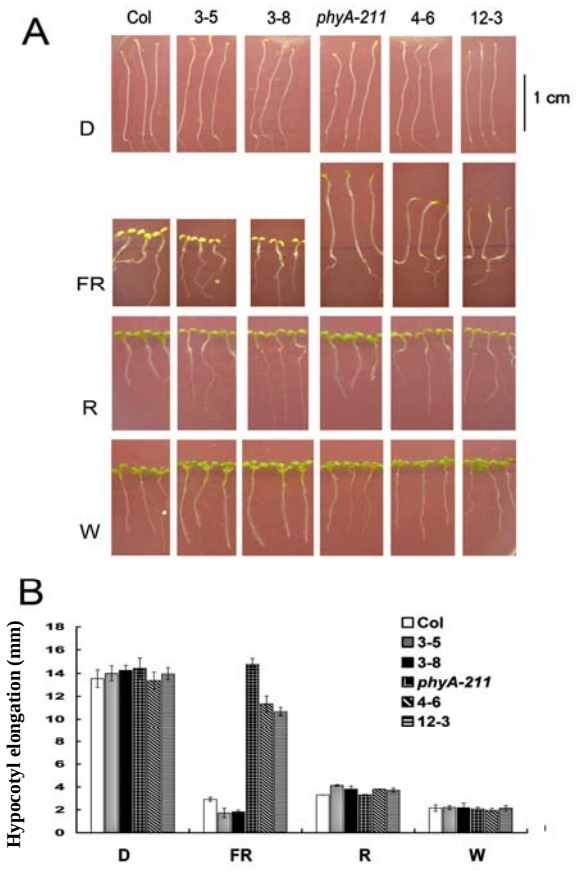

Fig. 2. Hypocotyl elongation in *Arabidopsis* plants expressing rice *PHYA* under different light conditions.
A. Overview of the phenotypes of WT, *phyA-211* mutant and transgenic seedlings (*35S::EYFP-OsPHYA*/Col and *35S::EYFP-OsPHYA*/*phyA-211*);
B. Quantification of hypocotyl elongation. one–day-old dark-grown seedlings were treated for 5 days under darkness (D), continuous FR light (FR, 2 μmol m^{-2} s^{-1}), continuous R light (R, 28 μmol m^{-2} s^{-1}) and white light (W, 100 μmol m^{-2} s^{-1}) after vernalization. WT: Col; *35S::EYFP-OsPHYA*/Col transgenic plants: 3-5 and 3-8; *phyA-211* mutant: *phyA-211*; *35S::EYFP-OsPHYA*/*phyA-211* transgenic plants: 4-6 and 12-3 (the same below). Each experiment was repeated at least three times. Mean values were calculated from at least 50 seedlings per genotype. Error bars represent standard deviation.

Seed germination under very low fluence and deep shade light: To further evaluate the function of rice phyA in *Arabidopsis*, we investigated the germination rate under VLFR, deep shade light, and pulses of deep shade light. WT, *phyA-21l*, *35S::EYFP-OsPHYA*/Col, and *35S::EYFP-OsPHYA*/*phyA-211* seeds were exposed to a FR light pulses to establish a Pfr/Pr cycle after imbibition. The FR light pulses of 0.04, 0.5, and 2.0 μmol m^{-2} s^{-1} were selected because VLFR occurs below Pfr/Pr = 0.1%. Seed germination in *Arabidopsis* can be triggered by PHYA-dependent VLFR. In *phyA-211* mutant seeds, FR pulses reduced the germination rate to 30% level in WT (Fig. 3A). This indicated that VLFR is the inductive response mediated by phyA (Botto *et al*., 1996). The observation that *35S::EYFP-OsPHYA*/*phyA-211* has the same germination ratio as WT indicated that the exogenous phyA has the same biological activity as *Arabidopsis* phyA with regard to mediating VLFR. The highest germination rate (about 90%) was observed in *35S::EYFP-OsPHYA*/Col, indicating that both *Arabidopsis* phyA and rice phyA were required for FR-induced germination in VLFR mode.

Arabidopsis seedlings are sensitive to variations in the R/FR ratio of incident light. They show shade-avoidance syndrome in response to reductions in the R/FR ratio (Ballare, 2009). WT, *phyA-21l*, *35S::EYFP-OsPHYA*/Col, and *35S::EYFP-OsPHYA*/*phyA-211* seeds showed very similar trends when exposed to deep shade light (R/FR=0.04) (Fig. 3B). However, the germination rate of *phyA-211* was only 30% on the second day of deep shade light treatment and remained at about 60% after the third day. In contrast, WT, *35S::EYFP-OsPHYA*/Col, and *35S::EYFP-OsPHYA*/*phyA-211* seeds showed high germination rates. The two transgenic lines reached 100% on the third day. These results suggest that exogenous rice phyA can fully replace *Arabidopsis* phyA and has the same biological activity in mediating shade-avoidance response.

Pulses of deep shade light were used to determine whether rice phyA is involved in the treatment predicted to establish very low proportions of Pfr. In the dark, the germination rate of WT, *35S::EYFP-OsPHYA*/Col, and *35S::EYFP-OsPHYA*/phyA-211 was about 60% (Fig. 3C). A single pulse of deep shade light strongly promoted germination in these seeds with *Arabidopsis* phyA and rice phyA. The germination rates of the two transgenic types were around 100%, indicating that exogenous rice phyA promotes seed germination in *Arabidopsis* during the pulses of deep shade light. On the contrary, regardless of pulses of deep shade light, the germination rate of *phyA-211* was only 40%, most likely because of the absence of phyA (Fig. 3C). This observation was consistent with the conclusion that VLFR is involved in the promotion of seed germination by deep shade light as described above and further indicated that rice phyA can replace *Arabidopsis* phyA in this capacity.

De-etiolation assays under red light pulses: It has been reported that POR enzymes (protochlorophyllide oxidoreductases) need high-energy light to catalyze the synthesis of chlorophyll and that the level of protochlorophyllide is reduced under FR light (continuous and pulses), preventing the synthesis of chlorophyll (Kneissl *et al*., 2008). Taking this so-called 'far-red light killing effect' into consideration, we examined the de-etiolation ability of *phyA-21l*, WT, *35S::EYFP-OsPHYA*/Col, and *35S::EYFP-OsPHYA*/*phyA-211* under red light pulses. Under control conditions (without red pulses), we counted the number of seedlings with unfolded cotyledons after the transfer from dark to light and used these as de-etiolation standards. The de-etiolation ability of *phyA-211* dark-grown seedlings declined faster than other dark-grown lines (Fig. 4A), suggesting that phyA plays a role in recovering from etiolation. Dark-grown WT and transgenic seedlings showed a slow downtrend in de-etiolation (Fig. 4A). On the eighth day, the proportion of de-etiolated *phyA-211* fell to 20%. In contrast, proportions of de-etiolation in WT and both types of transgenic seedlings remained at nearly 60%. This suggests that exogenous rice phyA can replace *Arabidopsis* phyA in the function of recovering green.

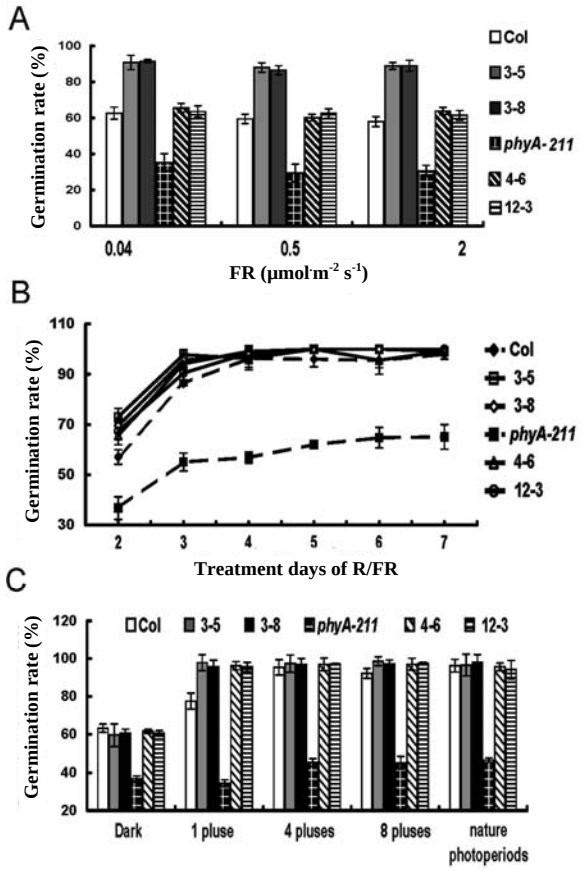

Fig. 3. Germination of *Arabidopsis* plants expressing rice *PHYA* under different treatments.
A. Germination rate of WT, *phyA-211* mutant and transgenic seedlings (*35S::EYFP-OsPHYA*/Col and *35S::EYFP-OsPHYA*/*phyA-211*) under different FR fluences. Seeds were incubated for 3 days at 7 °C followed by 8 hours at 35 °C. They were then exposed to FR light pulses. Germination was recorded after 4 days.
B. Germination rates of WT, *phyA-211* mutant and transgenic seedlings (*35S::EYFP-OsPHYA*/Col and *35S::EYFP-OsPHYA*/*phyA-211*) under the deep shade light (R/FR ratio = 0.04) on different days.
C. Germination rates of WT, *phyA-211* mutant and transgenic seedlings (*35S::EYFP-OsPHYA*/Col and *35S::EYFP-OsPHYA*/*phyA-211*) under the deep shade light pulse (R/FR ratio = 0.04). Each pulse occurred after 3 hours of imbibition. Chart shows four pulses total, one per day (the first pulse after 3 hours of imbibition) and eight pulses total, two per day. Mean values were calculated from at least 100 seeds per genotype. Error bars represent standard deviation.

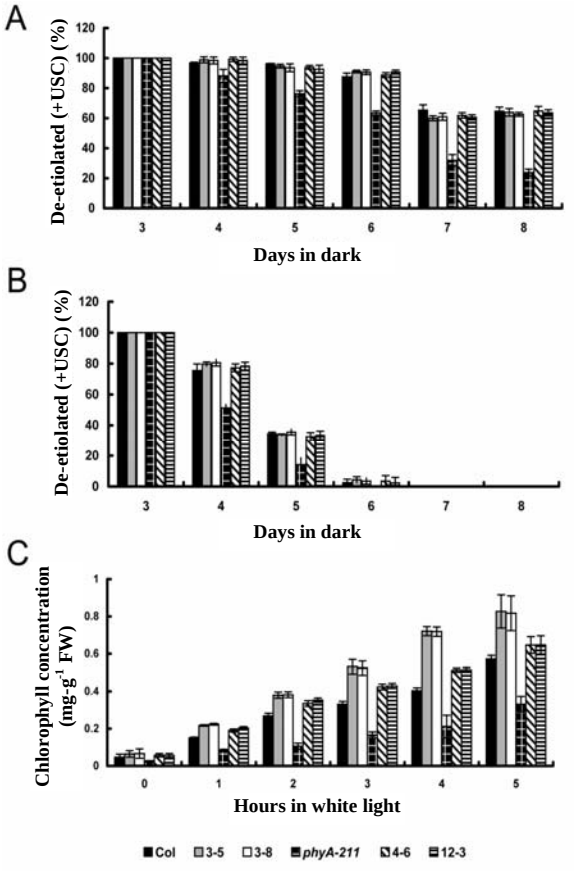

Fig. 4 De-etiolation assay of *Arabidopsis* plants expressing rice *PHYA* under different light conditions.
A, B. Recovery of etiolated seedlings upon transfer to white light. WT, *phyA-211* mutants and transgenic seedlings (*35S::EYFP-OsPHYA*/Col and *35S::EYFP-OsPHYA*/*phyA-211*) were grown in MS medium with (A) or without (B) sucrose in the dark for different lengths of time and then transferred to white light for 3 days.
C. Potentiation of rapid greening by a pulse of red light. 4-day-old dark-grown seedlings of WT, *phyA-211* mutant and transgenic seedlings (*35S::EYFP-OsPHYA*/Col and *35S::EYFP-OsPHYA*/*phyA-211*) were treated with a pulse of red light before transfer to continuous white light. Chlorophyll content was measured at different times following transfer to white light. Mean values were calculated from at least 50 seedlings per genotype. Error bars represent standard deviation.

During the de-etiolation recovery process, etiolated seedlings can also be de-etiolated by external factors, such as sucrose in the medium (Suc). In the absence of sucrose, dark-grown *phyA-211* seedlings lost the ability to de-etiolate after shorter times in the dark than WT seedlings (Fig. 4B). However, WT and both transgenic types lost their ability to de-etiolate after 6 days of darkness. It is suggested that the presence of sucrose in the medium delay the loss of de-etiolation ability in these seedlings. The same results were observed in experiments involving maltose. Surprisingly, all lines cultivated in the medium with glucose reached 100% de-etiolation regardless of the number of days in the dark (data not shown). This showed that both PHYA and carbohydrates may be essential for recovery from etiolation.

The accumulation of chlorophyll is an indispensable step in the de-etiolation recovery process. Using red light pulses, we examined chlorophyll accumulation in *phyA-21l*, WT, *35S::EYFP-OsPHYA*/Col, and *35S::EYFP-OsPHYA*/*phyA-211*. After a red pulse, the chlorophyll content of all lines tested exhibited a gradual uptrend correlated with duration of white light treatment. However, chlorophyll content in *phyA-211* mutant seedlings was consistently lower than in WT seedlings (Fig. 4C). These results are consistent with the de-etiolation experiment described above. *35S::EYFP-OsPHYA*/Col seedlings showed the most profound results in every treatment, suggesting that exogenous rice phyA may have an advantage over the endogenous *Arabidopsis* phyA in increasing chlorophyll content

during recovery. Although there were differences in chlorophyll content between all lines tested, the various lines showed similar responses to pretreatment with potential red-light pulses. Chlorophyll content increased in *phyA-21l*, WT, *35S::EYFP-OsPHYA*/Col, and *35S::EYFP-OsPHYA*/*phyA-211* seedlings, indicating that red light pulses are effective in promoting de-etiolation.

Hypocotyl elongation, petiole elongation, and flowering time under end-of-day far-red light treatments: End-of-day far-red light (EOD-FR) treatment includes a pulse of FR light before dark treatment begins. The plants treated with EOD-FR displayed similar developmental responses to those under continuous photoperiod with low R:FR (Dubois *et al*., 2010) . In order to survey the responses of *phyA-21l*, WT, *35S::EYFP-OsPHYA*/Col, and *35S::EYFP-OsPHYA*/*phyA-211* to EOD-FR treatment, hypocotyl elongation, petiole elongation, and flowering time were investigated with and without EOD-FR treatment. The hypocotyl lengths of WT plants grown under control conditions were shorter than those of plants under EOD-FR conditions, suggesting that EOD-FR reduces the inhibition of hypocotyl elongation (Fig. 5A and 5B). A similar effect of EOD-FR treatment was observed in *phyA-21l*, *35S::EYFP-OsPHYA*/*phyA-211* and *35S::EYFP-OsPHYA*/Col seedlings (Fig. 5B), indicating that EOD-FR treatment leads to reduced inhibition of hypocotyl elongation in all seedlings. Interestingly, *phyA-21l* mutant seedlings had longer hypocotyls than other seedlings under both EOD-FR treatment and control conditions, suggesting that *Arabidopsis* phyA and rice phyA play a role in the inhibition of hypocotyl elongation regardless of EOD-FR.

Like hypocotyl elongation, petiole elongation in *phyA-21l*, WT, *35S::EYFP-OsPHYA*/Col, and *35S::EYFP-OsPHYA*/*phyA-211* seedlings was also promoted by EOD-FR treatment (Fig. 5C). In contrast, petiole elongation of *35S::EYFP-OsPHYA*/Col and *35S::EYFP-OsPHYA*/*phyA-211* seedlings showed a larger and more obvious increase than that of *phyA-211* under EOD-FR conditions. This indicated that both *Arabidopsis* phyA and rice phyA are effective in restoring petiole elongation in response to EOD-FR treatment.

A low R/FR ratio can accelerate flowering as part of the shade-avoiding response (Kneissl *et al*., 2008). In our experiment, flowering time was measured by using the number of rosette leaves at first flower formation. In *phyA-21l*, WT, *35S::EYFP-OsPHYA*/Col, and *35S::EYFP-OsPHYA/phyA-211* seedlings, flowering time was greatly delayed in response to EOD-FR treatment (Fig. 5D). Moreover, this EOD-FR response became exaggerated in transgenic *35S::EYFP-OsPHYA*/Col seedlings. WT seedlings displayed nearly the same flowering time as *phyA-21l* seedlings under control conditions and flowered earlier than *phyA-21l* under EOD-FR treatments. The same results appeared in the comparison of *35S::EYFP-OsPHYA*/Col and *35S::EYFP-OsPHYA/phyA-211*, indicating that endogenous phyA play a role in the regulation of flowering time under EOD-FR treatment conditions. WT seedlings flowered later than *35S::EYFP-OsPHYA*/Col seedlings under EOD-FR treatment conditions, as with *phyA-21l* and *35S::EYFP-OsPHYA/phyA-211*. This suggests that rice phyA has the same function as *Arabidopsis* phyA in the regulation of flowering time under EOD-FR.

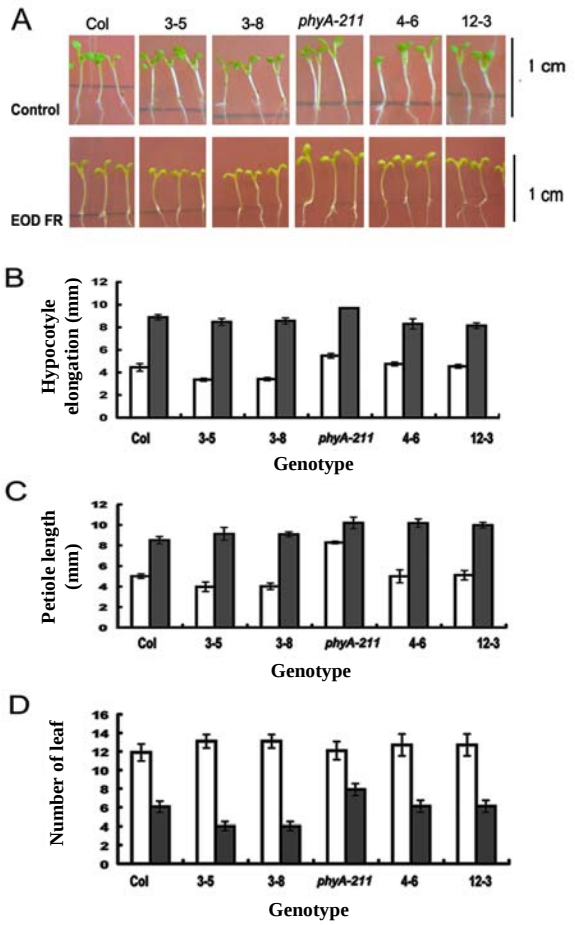

Fig. 5. Effects of EOD-FR treatments on *Arabidopsis* plants expressing rice *PHYA*.
A. Overview of the phenotypes of WT, phyA-211 mutant, and transgenic seedlings (*35S::EYFP-OsPHYA*/Col and *35S::EYFP-OsPHYA*/*phyA-211*).
B. Quantification of hypocotyl elongation. Seedlings germinated for 2 days under continuous W were transferred to photoperiods (9 hours W/15 hours D) or to EOD-FR (9 hours W + 10 minutes FR/14.75 hours D) for a further 7 days. Each experiment was repeated at least three times. Mean values were calculated from at least 50 seedlings per genotype. Error bars represent standard deviation.
C. Measurement of the length of petiole elongation. Seedlings were grown for 5 days under continuous W and transferred to photoperiods (9 hours W/15 hours D) or to EOD-FR (9 hours W + 10 minutes FR/14.75 hours D) until plants flowered. Mean values were calculated from at least 20 seedlings per genotype. Error bars represent standard deviation.
D. Flowering time determined by number of rosette leaves at inflorescence process. Seedlings were grown for 5 days under continuous W and transferred to photoperiods (9 hours W/15 hours D) or to EOD-FR (9 hours W + 10 minutes FR/14.75 hours D) until plants flowered. Mean values were calculated from at least 20 seedlings for each genotype. Error bars represent standard deviation

Discussion

Monocot plants evolved from herbal dicot plants. This raises the question of whether monocot phyA can replace the function of dicot phyA. To date, all studies on oat *PHYA* and rice *PHYA* expression in tobacco and *Arabidopsis* have attempted to answer this question via

the inhibition of hypocotyl elongation under different light conditions (Stockhaus *et al*., 1992; Boylan *et al*., 1994; Emmler *et al*., 1995; Jordan *et al*., 1995; Jordan *et al*., 1997; Halliday *et al*., 1999; Casal *et al*., 2002; Kneissl *et al*., 2008). However, other studies have different point of emphasis. Our results suggest that rice phytochrome A expressed in *Arabidopsis* plays a role in several physiological responses related to seed germination, hypocotyl elongation, growth, greening of the etiolated seedlings, and seedling responses to end-of-day far-red light treatment.

We confirmed that exogenous rice phyA was biologically active in *Arabidopsis* by using the inhibition of hypocotyl elongation by prolonged R light or FR light as a simple and repeatable assay for phytochrome action (Halliday *et al*., 1999). The expression of *OsPHYA* in the *phyA-211* mutants had no visible effect on hypocotyl elongation in dark-grown seedlings but it slightly enhanced the FR-mediated inhibition of hypocotyl elongation and significantly reduced the R- and W-mediated hypocotyl elongation. These results indicated that at least some exogenous rice phyA are chromophorylated and active in all of the transgenic lines, as indicated in previous reports (Halliday *et al*., 1999). PhyA is a FR-light sensor and responsible for the FR HIR. It has been reported that overexpression of *OsPHYA* would lead to a significant enhancement of the FR HIR controlling hypocotyl elongation (Emmler *et al*., 1995; Halliday *et al*., 1999; Kneissl *et al*., 2008). However, no such enhancement was observed in our experiments (Fig. 2B). The expression of *OsPHYA* in the *phyA-211* mutant slightly enhanced the inhibition of hypocotyl elongation. This HIR-related distinction was probably due to the extent of exogenous *OsPHYA* expression or to structural differences between rice and *Arabidopsis* phyA.

In most reported cases, phyA and phyB showed discrete photosensory functions, whereby phyA mediated plant responses to prolonged FR light and acted non-photoreversibly during induction reactions and phyB mediated responses to prolonged R light and acted photoreversibly under inductive conditions (Lagarias *et al*., 1997). PhyB was the predominant form in light-grown tissue, remained stable in the light, and mediated low-fluence response (LFR) to continuous red light and pulsed red light (Reed *et al*., 1993; Robson *et al*., 1993; Halliday *et al*., 1994). In this way, phyB was also the primary mediator of shade avoidance, with other light-stable forms in *Arabidopsis*, such as phyC, phyD and phyE (Franklin, 2008; Ballare, 2009). It was confirmed that not only WT and *phyA-211* mutant seedlings but also transgenic seedlings showed identical inhibition of hypocotyl elongation under continuous red light. The shortened hypocotyl was also observed under White light. This implys that the synergistic effects of different phytochromes are more effective on the inhibition of hypocotyl elongation. Rice phyA also could act as a R-light sensor for hypocotyl elongation in the absence of phyB (Halliday *et al*., 1999; Kneissl *et al*., 2008). However, in the presence of phyB, the effects of OsphyA were unremarkable.

Photoresponses involving phytochrome A were clearly visible in both dark-grown and green plants in the very-low-fluence-response (VLFR) and high-irradiance-response (HIR) (Quail *et al*., 1995; Casal *et al*., 1998). In *Arabidopsis*, phytochrome A induced seed germination under VLFR and heavy canopy shade (R/FR<0.05) in the field (Dechaine *et al*., 2009). In addition, phyA promoted seed germination at warmer temperatures (Heschel *et al*., 2007). In this study, the seed germination of *35S::EYFP-OsPHYA*/*phyA-211* achieved the same level of WT under VLFR conditions (Fig. 3A). Exogenous rice phyA seemed to promote seed germination in the VLFR mode. Although we proved the effects of rice phyA on seed germination in the VLFR mode, it remained to be seen whether rice phyA had the same function under other conditions. Continuous and pulsed deep shade light had a slight effect on *phyA-211* mutant seeds because of the absence of phyA (Fig. 3B and 3C). However, the germination rate of WT and transgenic seeds still reached about 100%. These data suggest that exogenous rice phyA can induce seed germination under VLFR and deep shade light, like endogenous phyA.

Dark-grown seedlings display a skotomorphogenic phenotype. During de-etiolation, phytochromes perform overlapping functions, in combination with cryptochrome UV-A/blue light photoreceptors Cry1 and Cry2 (Franklin & Quail, 2010). Some deficiency has been observed in the greening process during de-etiolation in *phyA-211* mutants (Tepperman *et al*., 2006). Compared with WT seedlings, *phyA-211* mutants lost the ability to de-etiolate quickly. This gave a sufficient explanation of the unique role of phyA in mediating de-etiolation (Parks & Quail, 1993; Whitelam *et al*., 1993). Moreover, the results showed that carbohydrate in the medium enhanced the de-etiolated ability of all etiolated seedlings. The fact that carbohydrates had the same effect as phyA was due to their roles as substrates in chlorophyll biosynthesis. The proportion of de-etiolated *35S::EYFP-OsPHYA*/*phyA-211* plants was the same as that of WT, suggesting that exogenous rice phyA can replace *Arabidopsis* phyA in recovering the de-etiolation ability (Fig. 4A and 4B). It has been documented that phyA regulated the *LHCB* genes that encode chlorophyll a/b-binding proteins during de-etiolation (McCormac & Terry, 2002). Our results further confirmed that exogenous rice phyA had the same function as endogenous *Arabidopsis* phyA in mediating the synthesis of chlorophyll.

End-of-day far-red light (EOD-FR) treatment is composed of a pulse of FR given at subjective dusk (Kasperbauer, 1971). This triggers a circadian-clock-gated response (Salter *et al*., 2003). It has been shown that *phyA*/*phyB* double mutants responded to low R/FR ratio signals and EOD-FR treatments by a promotion of elongation growth and earlier flowering (Halliday *et al*., 1994; Devlin *et al*., 1996). EOD responses are greatly attenuated in *phyB*-null seedlings, indicating that phyB is the principal photoreceptor mediating growth responses to EOD-FR pulses (Whitelam & Devlin, 1997). Moreover, the already-elongated hypocotyls or petioles of *phyB* mutant seedlings show no further elongation in response to EOD-FR (Halliday *et al*., 1999). In our experiment, all

seedlings tested displayed a short-hypocotyl phenotype under control conditions and a long-hypocotyl phenotype in response to EOD-FR. This validated previous results that phyB played the primary role in mediating hypocotyl elongation responses to EOD-FR pulses. Under control conditions, both rice Pfr_A and *Arabidopsis* Pfr_A, present at the transition from light to darkness, caused an inhibition of hypocotyl elongation during the dark period. When treated with EOD-FR, the conversion of some Pfr_A to Pr_A reduced growth inhibition. This is consistent with data published by Halliday *et al*., (Halliday *et al*., 1999). Interestingly, *phyA-211* mutant seedlings had longer hypocotyls than other seedlings tested under both EOD-FR treatments and control conditions, suggesting that regardless of the form of Pfr_A and Pr_A, both *Arabidopsis* phyA and rice phyA play a role in the inhibition of hypocotyl elongation.

Petiole elongation and flowering time were assessed to establish whether exogenous rice phyA could function in other EOD-FR responses. In contrast to hypocotyl elongation, the restoration of petiole elongation in *35S::EYFP-OsPHYA*/*phyA-211* seedlings was obvious, indicating that rice phyA operated differently in these two organs. Moreover, under EOD-FR treatment conditions, petiole elongation in *35S::EYFP-OsPHYA*/Col and *35S::EYFP-OsPHYA*/*phyA-211* showed a larger and more obvious increases than that in *phyA-211* mutants. These data suggest that both rice phyA and *Arabidopsis* phyA might restore the petiole elongation response to EOD-FR, but exogenous rice phyA might play a more important role.

Flowering time in *A. thaliana* is responsive to many environmental cues, including photoperiod, vernalization, ambient temperature, and nutrient status (Engelmann & Purugganan, 2006). Molecular studies have conclusively shown that phyA, phyB, phyC, and phyD alter flowering time (Aukerman *et al*., 1997; Maloof *et al*., 2001; Balasubramanian *et al*., 2006; Filiault *et al*., 2008). However, phyB plays a major role in the regulation of flowering time in *Arabidopsis* (Halliday *et al*., 1999). Overexpression of *OsPHYA* largely reduced the flowering time of WT under EOD-FR treatments. This characteristic early-flowering phenotype of *35S::EYFP-OsPHYA*/Col suggested that both endogenous *Arabidopsis* phyA and exogenous rice phyA may be involved in flowering responses in *Arabidopsis*. In addition, the phenomenon of close flowering time in WT and *35S::EYFP-OsPHYA*/*phyA-211* seedlings under EOD-FR treatment conditions indicated that a heterologous phyA may replace endogenous phyA in flowering responses. However, rice phyA functioned differently in different EOD-FR responses, possibly due to organ-specific differences in expression.

Conclusions

Rice phytochrome A in *Arabidopsis* plays varied role in many physiological responses. The differences observed here may be due to different levels of expression in different parts of *Arabidopsis* seedlings or to the different absorption spectra required during different developmental stages. To determine whether monocot phyA could replace dicot phyA, we used only phyA of rice and *Arabidopsis*. We found that rice phyA can replace *Arabidopsis* phyA in many physiological responses. Further research will focus on the function of other monocot phyAs in various dicots in order to elucidate the difference of phytochromes and their signal transduction pathway in monocots and dicots. Taken together, our results suggest that, for the most part, rice phyA can substitute for *Arabidopsis* phyA in regulating a number of common responses despite their different structures and intrinsic mechanisms of action.

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