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Phytochrome-mediated growth inhibition of seminal roots in rice seedlings

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Abstract

In rice (*Oryza sativa*) seedlings, continuous white-light irradiation inhibited the growth of seminal roots but promoted the growth of crown roots. Here, we examined the mechanisms of photoinhibition of seminal root growth. Photoinhibition occurred in the absence of nitrogen, but increased with increasing nitrogen concentrations. In the presence of nitrogen, photoinhibition was correlated with coiling of the root tips. The seminal roots were most photosensitive 48 - 72 h after germination during the 7-d period after germination. White-light irradiation for at least 6 h was required for photoinhibition, and the Bunsen-Roscoe law of reciprocity was not observed. Experiments with phytochrome mutants showed that far-red light was perceived exclusively by phyA, that red light was perceived by both phyA and phyB, and that phyC had little or no role in growth inhibition or coiling of the seminal roots. Fluence-response curve analyses also showed that phyA and phyB control very low fluence response and low fluence response, respectively, in the seminal roots. This was essentially the same as the growth inhibition previously observed at the late stage of coleoptile development (80 h after germination). These results also suggest that other blue-light photoreceptors are involved in growth inhibition of the seminal roots. The photoperceptive site for the root growth inhibition appeared to be the roots themselves. All three phytochrome species of rice were detected immunochemically in roots.

Abbreviations: LFR, low-fluence response; VLFR, very low-fluence response.

Introduction

Higher plants change their growth extent and degree in response to various environmental stimuli, such as gravity, light, moisture, nutrients, temperature, and obstacles to adapt to their environment and gain maximum advantage for growth. Not only shoots but also roots respond to their light environment and change their growth and development: Light irradiation affects rate (Kurata and Yamamoto 1997, Correll and Kiss 2005) and direction (Okada and Shimura 1992, Takano et al. 2001, Kiss et al. 2002) of root growth and development of root hairs (De Simone et al. 2000). Growth of rice seminal roots has been known to be inhibited by light irradiation for ~40 years (Ohno and Fujiwara 1967). Exposure to blue, red and far-red light inhibited root growth to similar extents, suggesting that phytochrome acts a photoreceptor. It is likely that the photoperception sites are on the roots because light exposure only to shoots did not result in growth inhibition in roots. In the case of red and far-red light irradiation, growth inhibition appeared to be due to inhibition of cell elongation rather than inhibition of cell division.

Using rice mutants deficient in each of the three phytochromes in rice, phyA, phyB and phyC (Takano et al. 2005), we previously examined photoinhibition of coleoptile growth at different developmental stages (Xie et al. 2007). At the early stage of development (40 h after germination), photoinhibition was predominantly due to the phyB-mediated low-fluence response (LFR), but at the late developmental stage (80 h after germination), it consisted of the phyA-mediated very-low-fluence response (VLFR) as well as the

phyB-mediated LFR (Xie et al. 2007). LFR is typically induced in the photon fluence range of 1 – 1000 $\mu\text{mol m}^{-2}$, while VLFR is typically induced in the range of 0.1 – 1 $\mu\text{mol m}^{-2}$ (Shinomura et al. 1996, Schäfer and Nagy 2006). Because growth of the seminal roots is also affected by light (Ohno and Fujiwara, 1967), we decided to carry out a similar study on rice roots to determine which of the three phytochrome species was responsible for photoregulation of root growth. We found that photoinhibition of root growth increased with increasing nitrogen concentration in culture medium. Furthermore, phyA and phyB functioned in the growth inhibition of the seminal roots in a similar manner to growth inhibition previously observed in coleoptiles at their late developmental stage.

Materials and methods

Plant materials and growth conditions

Dehusked rice seeds (*Oryza sativa* L. cv. Nipponbare) were surface sterilized, placed on 0.2% gellan gum containing half concentration of Murashige and Skoog salt mixture (MS; Murashige and Skoog 1962) in glass tubes, and grown for 7 days at 28°C. The 1/2 MS salts contained 30.0 mM total nitrogen. The *phyA* (Takano et al. 2001) and *phyC* (Takano et al. 2005) mutants are insertional mutants of a rice retrotransposon *Tbs17*, and were isolated by PCR-based screening. The *phyB* mutant was obtained by screening γ ray-mutagenized population for an elongated-coleoptile phenotype (Takano et al. 2005). In experiments of local light irradiation, sterilized seeds were placed on medium which was covered with sheets of aluminum foil. Furthermore, the surface of the

gellan gum medium was overlaid by a 0.5-mm-thick layer of carbon-black containing medium. Seedlings were exposed to continuous white light or kept in darkness for 7 days.

Light sources

White light ($55 \mu\text{mol m}^{-2} \text{s}^{-1}$) was supplied by white fluorescent tubes (FL20W-B, Hitachi, Tokyo) or high intensity discharge lamps (MLBOC400C-U, Mitsubishi, Tokyo). Blue light ($14 \mu\text{mol m}^{-2} \text{s}^{-1}$) was supplied by blue fluorescent tubes (FL-20S-B, Toshiba, Tokyo) filtered through a blue acrylic sheet (3 mm thickness; Acrylight K5-302, Mitsubishi Rayon, Tokyo). Red light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$) was supplied by red fluorescent tubes (FL-20S, Re66, Toshiba) filtered through a red acrylic sheet (3 mm thickness; Acrylite K5-102, Mitsubishi Rayon). Far-red light ($38 \mu\text{mol m}^{-2} \text{s}^{-1}$) was supplied by far-red fluorescent tubes (FL-20FR-74, Toshiba) filtered through a black acrylic sheet (3 mm thickness; Deraglass A-900, Asahi Kasei, Tokyo; Hanzawa et al. 2002). The blue acrylic filter exhibited the transmission peak at 458 nm with a half-band width of 74 nm. The emission peaks of the red and far-red fluorescent tubes were at 658 and 741 nm, respectively; the red and black acrylic sheets were long wavelength pass filters of which the wavelengths of 50% of peak transmittance were 609 and 738 nm, respectively. All these light sources were further combined with the dispersion filters (3 mm thickness; Acrylite K5-001E, Mitsubishi Rayon)

Protein blot analysis

Soluble protein was extracted from 3-day-old dark-grown or light-grown seedlings by using protein extraction buffer (100 mM Tris-HCl, pH 8.3, 5 mM EDTA, 0.2%

2-mercaptoethanol, and 100 mM phenylmethylsulfonyl fluoride) and precipitated with 60% saturated ammonium sulfate (Nagatani et al. 1993). The precipitated material was resuspended in protein extraction buffer and protein concentrations were determined by using Coomassie PLUS Protein Assay Reagent (Pierce, Rockford, IL). Sixty µg of protein were size-fractionated by SDS-PAGE in 10% gel and then blotted onto PVDF membrane (Millipore, Billerica, MA). Immunochemical detection was performed by the use of PHYA, PHYB and PHYC-specific antibodies as described in Takano et al. (2005). PHYA-specific antibody was the anti-rye phytochrome monoclonal antibody (mAR07; Takano et al. 2001). PHYB- and PHYC-specific antibodies were conventional rabbit antibodies against the C-terminal half of PHYB and PHYC, respectively, expressed in *Escherichia coli* (Takano et al. 2005).

Nutrient treatment

The MS salt contains NH_4NO_3 and KNO_3 with a molar ratio of 1.10:1. The MS media containing various nitrogen concentrations from 0 to 60 mM were prepared by adding both salts to the nitrogen-free 1/2 MS medium with a molar ratio of 1.10.

Results

Effects of nitrogen and light on growth of the seminal roots

Wild-type rice seedlings were grown in different conditions for 7 days to know whether either nitrogen concentration of the growth media or light irradiation

affected the growth of their seminal and crown roots. When seedlings were grown in darkness on half-strength MS medium that contained 30 mM nitrogen, the seminal roots grew longer than 80 mm. In contrast, crown roots were short (Fig. 1A and F). Under the continuous white-light irradiation the seminal roots started coiling when they grew approximately 20 mm long, and eventually stopped growing (Fig. 1B and E). Growth of the seminal roots was thus inhibited by light exposure by about two thirds. Growth of the crown roots was, however, promoted by white light condition by about two-fold (Fig. 1B and F). When seedlings were grown on the nitrogen-free medium, both the seminal and crown roots grew longer than those at 30 mM nitrogen, and light-induced growth modulation was observed to a less extent than that observed at 30 mM nitrogen (Fig. 1C, D and F). Though growth of both the seminal and crown roots was affected by the light condition, we further investigated effects of light exposure on growth of the seminal roots here.

First we examined the effects of nitrogen concentration of the medium on growth of the seminal roots in the dark and light conditions, raising rice seedlings on the medium containing 0 to 60 mM nitrogen (Fig. 2A). Growth of the seminal roots was increasingly inhibited by nutrient nitrogen up to 0.5 mM in the dark, but it was almost constant at nitrogen concentrations higher than 0.5 mM. Growth of the seminal roots in the light condition was always smaller than that in the dark. Relative growth inhibition by light irradiation (that is, the decrease in root length in the light divided by root length in the dark) was almost constant (~30%) at nitrogen concentrations up to 0.5 mM (Fig. 2B). When nitrogen concentrations were increased over 0.5 mM, relative growth inhibition gradually increased and reached ~70% at 30 and 60 mM nitrogen. Thus, light-induced

growth inhibition was maximal at higher concentrations of nutrient nitrogen in the seminal roots. Coiled root tips were observed only when rice seedlings were grown in the light condition and in the presence of the nutrient nitrogen at concentrations more than 0.1 mM. The frequency of the coiled root tips was almost inversely proportional to growth of the seminal roots in the light condition (Fig. 2C).

In *Arabidopsis*, submillimolar concentrations of L-glutamate inhibit growth of the primary roots with a distorted root-tip morphology (Walch-Liu et al., 2006). The shape of the root tip is somewhat similar to the coiled root tips of rice seedlings reported here. Therefore, we examined the effects of Glu on rice root growth. Though 50 mM Glu did inhibit growth of the seminal roots of rice seedlings by 60%, which was similar to inhibition by total nutrient nitrogen in the light condition (Fig. 2A), it did not induce coiling of the seminal root (data not shown). In the case of the Glu-induced growth inhibition of *Arabidopsis*, the activity of the root apical meristem is reduced and its area is caused to shrink (Walch-Liu et al., 2006). We examined root apical meristem of rice seedlings after staining amyloplasts in the root cap with I₂/KI. No apparent differences in morphology were observed between coiled and normal root tips (data not shown).

Effects of duration and intensity of light treatment

To determine when white light inhibited growth of the seminal roots most efficiently, seedlings were exposed to white light only for 24 h during the 7-day-long growth period. White-light irradiation on the 1, 6 or 7th day only slightly inhibited elongation of the seminal root. The shortest seminal roots were observed when white-light irradiation was carried out on the third day

(48-72 h) after germination (Fig. 3A).

Next we examined how long white-light irradiation was needed in the most photosensitive period of growth determined above. Two-day-old etiolated seedlings were irradiated with different durations of continuous white light from 0.02 to 24 h, after which they were kept in darkness until the 7th day again. When treated for 1.5 h or shorter, the seminal roots were almost as long as those grown in total darkness. When seedlings were exposed to white light for longer than 6 h, the seminal roots were significantly shorter than those in the dark control ($P=0.035$ for the 6-h irradiation in t -test) (Fig. 3B). These results indicated that this growth inhibition required white-light irradiation for longer than 6 h even in the most photosensitive state.

To know whether this photoresponse obeyed the Bunsen-Roscoe law of reciprocity, two-day-old seedlings were exposed to white light for various times, but with the same total photon fluence (4.8 mol m^{-2}). Light irradiation for 3 h or shorter did not inhibit seminal root growth, indicating that inhibition of the seminal root growth did not follow the Bunsen-Roscoe law (Fig. 3C). Thus, at least several h of white-light irradiation were needed to inhibit the growth of seminal roots.

Growth inhibition in the phytochrome mutants

To identify photoreceptors involved in inhibition of growth of the seminal roots, we examined growth of the rice phytochrome mutants under continuous irradiation with white, blue, red or far-red light for 7 days (Fig. 4, top). In darkness only small differences were observed in root growth among the mutants and wild type. Growth inhibition of the seminal roots was observed under all light conditions

tested in the wild type. In contrast, no inhibition was observed in *phyA* single mutants or *phyA phyC* double mutants under the far-red light condition. The inhibitory effects of white, blue and red light were very similar between *phyA* single mutants and *phyA phyC* double mutants. On the other hand, the inhibitory effects of red light were partially lessened in *phyB* and *phyB phyC* to the same extent. However, root growth of the two mutants was inhibited by far-red light as effectively as it was in the wild type. Seminal root elongation was not inhibited in *phyA phyB* double mutants under red- and far-red-light conditions. The frequency of coiling in the seminal root tips (Fig. 4, bottom) seemed to be correlated with growth inhibition (Fig. 4, top). These results suggest that far-red light is perceived exclusively by phyA, that red light is perceived by both phyA and phyB, and that phyC has little or no effect on growth inhibition or coiling of the seminal roots. Though the growth of the *phyA phyB* seminal roots was not inhibited by red or far-red light, it was retarded in the blue-light condition (Fig. 4, top), suggesting that blue-light photoreceptors other than phytochromes were also involved in this response.

Dose response of red-light irradiation

We examined the dose response curves of red-light irradiation in *phyA* and *phyB* mutants (Fig.5). Red-light irradiation of 0.099 nmol m⁻² s⁻¹ or higher fluence rates for 24 h inhibited the seminal root elongation of wild type and *phyB* mutants in a similar manner. In contrast, the seminal root elongation of *phyA* mutants was not inhibited by red-light irradiation of up to 5100 nmol m⁻² s⁻¹. These results show that phyB required higher fluence rates to inhibit seminal root growth than phyA.

Effects of the local white-light irradiation.

To identify the photoperception site for inhibition of seminal root growth, only the above-ground portion of seedlings was treated with white-light irradiation. The partial light irradiation of shoots inhibited the seminal root elongation only about 14% (Fig. 6). This suggests that seminal roots perceive light and regulate their growth organ-autonomously, or that shoots are partially involved in this growth regulation. We examined the presence of phytochromes in roots with protein blot analysis by the use of the PHYA, PHYB and PHYC-specific antibodies. All the three phytochrome proteins were found in roots in both light and dark conditions (Fig. 7). Furthermore, levels of phyB were not dependent on light condition, while those of phyA and phyC were down-regulated by white light. These results are consistent with the fact that phyA and phyB belong to the light-labile and the light-stable phytochrome, respectively, in *Arabidopsis*, but are different from results of *Arabidopsis* phyC that is another light-stable phytochrome (Sharrock and Clack 2002).

Discussion

Plant root architecture is affected by a number of environmental conditions, especially by mineral nutrients (Osmont et al. 2007). Both nutrient nitrogen (Kawata et al. 1977, Tanaka et al. 1993) and light irradiation (Ohno and Fujiwara 1967) inhibit growth of the rice seminal roots. Recently Hirano et al. (2008) showed that the ammonium ion was responsible for this inhibition, excluding an

involvement of nitrate ion. Ohno and Fujiwara (1967) previously reported that red and far-red-light irradiation induced growth inhibition by inhibiting cell elongation, while blue-light irradiation inhibited both cell elongation and cell division. This suggests that the mode of growth inhibition is different between red and far-red light and blue light, which implicates the involvement of blue-light pigments other than phytochromes. In the present study, we examined the effects of nutrient nitrogen and light conditions on growth of seminal roots more closely, and found that light sensitivity for growth inhibition of the seminal roots was promoted by nutrient nitrogen in a dose-dependent manner when nitrogen was higher than 0.5 mM (Fig. 2A and B). Because the extent of light inhibition reached a plateau at nitrogen concentrations higher than ~10 mM (Fig. 2B), we examined the mode of light inhibition in the presence of 30 mM nitrogen (namely, in 1/2 MS medium). Investigation of phytochrome mutants clearly demonstrated that the photoreceptors responsible for the growth inhibition were mainly phyA and phyB. Far-red light was perceived solely by phyA, and red light was perceived by both phyA and phyB; phyC did not play a significant role. Blue-light pigments other than phytochromes also appeared to function in this growth inhibition (Fig. 4), as was observed previously (Ohno and Fujiwara 1967).

Light-induced growth inhibition was also observed in coleoptiles in rice seedlings (Pjon and Furuya 1967). The mode of growth inhibition in rice coleoptiles changes depending on the age of the seedlings. At the early stage of coleoptile development (coleoptile lengths: 3 - 5 mm) phyB predominantly functions by absorbing light shorter than red light. Far-red light does not affect coleoptile growth at this stage. In contrast, phyA acts responding to far-red light

and phyA and phyB work together for red light at the late stage; blue-light photoreceptors may be also involved (Xie et al. 2007). This indicates that the combination of functioning photoreceptors is similar in growth inhibition of the seminal roots and coleoptiles at the late stage. The mode of phytochrome action also appears similar between the two growth inhibiting responses in rice seedlings. In coleoptiles, phyB-regulated growth inhibition is classified as an LFR, while phyA-regulated growth inhibition is classified as a VLFR (Xie et al. 2007). In seminal roots, a photon fluence of $\sim 10 \mu\text{mol m}^{-2}$ red light was sufficient for phyA inhibition, while a photon fluence $> 10,000$ times this amount was needed for phyB inhibition (Fig. 5). This suggests that phyA and phyB also mediate VLFR and LFR, respectively, in the seminal roots. These results may be consistent with the fact that the late stage of coleoptile development (80 h after germination) occurs during the most photosensitive period of growth inhibition in the seminal roots (72 - 96 h after germination; Fig. 3A). On the other hand, there are marked differences between the two growth inhibitions observed in coleoptiles and seminal roots. The Bunsen-Roscoe relationship that was observed in coleoptiles did not hold in the seminal roots (Fig. 3C). Growth inhibition of coleoptiles occurred even after a brief light exposure (Pjon and Furuya 1967, Xie et al. 2007), while at least 6-h-long irradiation was necessary to inhibit the growth of seminal roots (Fig. 3B).

Experiments with local light irradiation show that the photoreceptors in roots are mainly responsible for growth inhibition of the seminal roots (Fig. 6). The smaller inhibiting effects of light exposure to shoots may be due to light conducted through vascular tissues from shoots to roots; far-red light has been shown to be the most efficiently transmitted light in stems and roots (Sun et al.

2005). The present result is consistent with the previous report that the photoperceptive site of growth inhibition of the seminal roots in two-day-old rice seedlings was the root itself (Ohno and Fujiwara 1967). Photoperception for white-light induced negative phototropism of maize roots occurred in the root cap (Mullen et al. 2002). In positive root phototropism of *Arabidopsis*, sensing of red light through phyA and phyB also occurs in the root itself (Kiss et al. 2003). The protein blot analysis indicates that all of the rice phytochromes are present in roots (Fig. 7). In *Arabidopsis* all 5 phytochromes were also found in roots by the use of green fluorescent protein- or luciferase-fused phytochromes (Salisbury et al. 2007). In fact, many photomorphogenetic responses have been reported in roots of various plant species (see De Simone et al. 2000, and references therein), and mechanisms underlying photoresponses in roots are being dissected in a molecular term (Molas and Kiss 2008, Boccalandro et al. 2008).

The seminal root tips always coiled when their growth was inhibited by nutrient nitrogen in the light condition (Fig. 2C). It seems worth noting that the seminal roots of *OsRAA1*-overexpressing rice plants grow more slowly, and form helices to various extents (Ge et al. 2004). Though the function of *OsRAA1* is unknown, *OsRAA1* appears to be involved in controlling the cell cycle, and to be responsible for limiting root growth by inhibiting the onset of anaphase (Han et al. 2008). However, the present results do not appear to show a causal relationship between growth inhibition and the occurrence of coiling in the seminal roots for two reasons. First, coiling did not occur in the dark condition even though growth of the seminal roots was inhibited by nitrogen in the dark, and second, the frequency of coiling was not correlated with the increase of relative growth inhibition that was observed at > 1 mM nitrogen in the light condition (Fig. 2B).

These results suggest that the molecular mechanisms for growth inhibition by nutrient nitrogen differ between the dark and light conditions.

Our results also show that light irradiation has an opposite effect on growth of the seminal and crown roots: light is inhibitory for the seminal roots, while it is promotive for the crown roots. In contrast, nutrient nitrogen always inhibits the growth of both types of roots (Fig. 1F). Light was found to promote growth in the *Arabidopsis* primary roots, where phyA and phyB were responsible for photoperception as well as photosynthetic activity (Kurata and Yamamoto 1997, Correll and Kiss 2005). Growth orientation of the crown roots is also affected by light exposure, while growth orientation of the seminal roots is controlled by the gravity vector irrespective of light conditions. The crown roots grow in a horizontal direction in the dark, while they grow towards the gravity vector in the light (Takano et al. 2001). The contrasting responses to light in the seminal and crown roots may help the transition of rice seedlings from the embryonic root system in which the seminal roots are predominant, to the fibrous root system that contains numerous crown roots (Hochholdinger et al. 2004).

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Figure legends

Fig. 1. Effects of nitrogen and light on the growth of roots of rice seedlings.

Seedlings were grown for 7 days under continuous white light (B and D) or in darkness (A and C) in the presence (A and B) or absence of 30 mM nutrient nitrogen ($\text{NH}_4^+ + \text{NO}_3^-$) (C and D). E, Close-up view of a coiled root tip of the seminal root. F, Root length of the seminal and crown roots of rice seedlings grown for 7 days in the dark (solid bar) or light condition (open bar). Arrows and arrowheads indicate the seminal and crown roots, respectively. Under the light condition in the presence of nutrient nitrogen (B), the seminal root formed a coiled root tip (arrow) and stopped growing. Seedlings were grown in glass tubes of 3.0-cm diameter.

Fig. 2. Effects of concentration of nutrient nitrogen of growth media on the growth of seminal roots in the light (open circles) and dark conditions (closed circles). A, Root length. B, Root length in the light condition relative to that in the dark. C, Frequency of coiled root tips. Seedlings were grown for 7 days on 0.2% gellan gum containing various concentrations of nitrogen. Values represent mean $\pm$ SD of at least 9 seedlings. Roots grown in the light were significantly shorter than those in the dark in each concentration of nutrient nitrogen ($P < 0.01$ in *t*-test).

Fig. 3. Effects of timing (A), duration (B) and fluence rate (C) of light irradiation on seminal root growth. (A) Seedlings were subjected to white-light irradiation for 24 h during a 7-day-long growth period in the dark. (B) Two-day-old etiolated

seedlings were exposed to continuous white light ($55 \mu\text{mol m}^{-2} \text{s}^{-1}$) for various durations. They were kept in darkness again until the 7th day. (C) Two-day-old etiolated seedlings were exposed to white light of the same fluence (4.8 mol m^{-2}) for various durations. They were kept in darkness again until the 7th day. High intensity discharge lamps were used for shorter irradiations from 0.48 to 1.44 h. Fluorescent lamps were used for longer irradiations. Values represent mean $\pm$ SE of three independent experiments, in which 10 to 20 seedlings were measured.

Fig. 4. Effects of light quality on growth of seminal roots (top) and occurrence of coiled seminal roots (bottom) in phytochrome mutants grown for 7 days. Root lengths are shown in wild type (WT), *phyA* and *phyB* single mutants and *phyA phyB*, *phyA phyC* and *phyB phyC* double mutants from left to right. Values represent means with a standard error of the mean of 4 – 5 independent experiments, in which about 12 seedlings were examined.

Fig. 5. Effects of different fluence rates of red-light irradiation on growth of seminal roots in wild-type (open circles) and *phyA* (closed circles) and *phyB* (triangles) mutants. Two-day-old etiolated seedlings were exposed to red-light with the indicated fluence rates for 24 h, and then kept in darkness again till the 7th day. Values represent mean $\pm$ SE of three independent experiments, in which 10 to 20 seedlings were used. A single asterisk shows a significant difference ($P < 0.05$ in *t*-test) between length of light-exposed roots and totally dark-grown roots in each genotype, and double asterisks show a difference with a *P* value of 0.072.

Fig. 6. Effects of local light irradiation on the growth of seminal roots.

Seedlings were grown under continuous white light or in darkness. The irradiation was confined to shoots by preventing ambient light from entering the growth medium. Mean $\pm$ SD was obtained from 10 to 20 seedlings.

Fig. 7. Levels of phytochrome proteins in shoots and roots of wild-type seedlings.

Seedlings were grown under continuous white light or darkness for 3 days. Sixty μ g each of soluble protein were subjected to a protein blot analysis by the use of PHYA, PHYB and PHYC protein-specific antibodies.

Dark

Light

A **B** **C**