Subcellular partitioning-dependent functional switching of *Arabidopsis* photoreceptor phytochrome B in response to brassinosteroids

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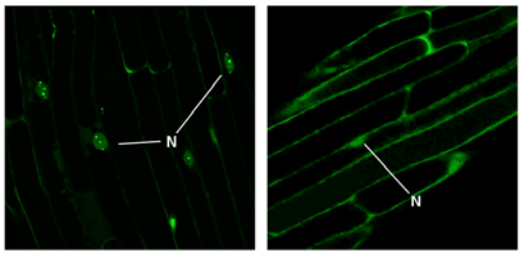
SYNOPSIS

Many organisms control their physiology and behavior in response to the local light environment, which is first perceived by photoreceptors that undergo light-dependent conformational changes. Phytochromes are one of the major photoreceptors in plants, controlling wide aspects of plant physiology by recognizing the light in red (R) and far-red (FR) spectra. Higher plants have two types of phytochromes; the photo-labile type I (phyA in Arabidopsis) and photo-stable type II (phyB-E in Arabidopsis).

Phytochrome B (phyB), a member of the type II phytochromes in *Arabidopsis*, shows classical R and FR reversibility between the inter-convertible photoisomers, Pr and Pfr. Interestingly, the Pr and Pfr isomers show partitioning in the cytosol and nucleus, respectively. In the over 50 years since its discovery, it has been thought that the type II phytochromes only function to mediate R light.

As described in the text, we have now discovered phyB has an active function in FR light. Even striking is that the R and FR light exert an opposite effect. Thus, FR light is not simply nullifying the R effect but has an opposing effect to R light. What is more interesting is that the phyB-mediated actions of FR and R light occur at different cellular compartment of the plant cell, cytosol and nucleus, respectively, which was proven through utilization of the cytosolic and nuclear-localized mutant versions of phyB.

Our observations thus shoot down a major dogma in plant physiology and will be considered highly provocative in phytochrome function. We argue that it would make much more sense that plants utilize the two isoforms rather than only one form, to effectively monitor the changing environmental light information and to incorporate the information into their developmental programs.



Keywords: brassinosteroid, seedling growth, red light, far red light, nucleo-cytoplasmic partitioning

Abstract

Introduction: Phytochromes are one of the major photoreceptors in plants, which recognize the light in red (R) and far-red (FR) spectra. phyB, a major type II phytochrome in *Arabidopsis*, exists as the two interconvertible photoisomers, the red light absorbing photoisomer (phyB-Pr; λmax, 660 nm) and the far-red light absorbing photoisomer (phyB-Pfr; λmax, 730 nm), which are partitioned in the cytosol and nucleus, respectively. phyB has long been regarded to mediate R light responses through nuclear-partitioned Pfr, whereas the cytosolic Pr is considered mostly null. On the contrary to this view, we tested the role of nuclear and cytosolic phyB in R and FR light.

Materials and Methods: Growth in plants is critically controlled by light and is in concert with endogenous growth regulating hormones such as brassinosteroids (BRs). We, thus, tested possible interaction of phyB and BRs in *Arabidopsis* seedlings. For this purpose, we employed an *Arabidopsis* mutant lacking phyB (*phyB-5*) and its isogenic wild type (*Ler*) along with transgenic plants overexpressing a phyB-GFP fusion protein and cytosolic and nuclear partitioned PBG(G767/R) and PBG(G767/R)-NLS. We examined BR-mediated seedling growth and gene expression responses of these plants under darkness and R and FR light.

Results and Discussion: In R light and FR light, phyB enhances and inhibits, respectively, seedling growth along with increasing concentration of BR in the media. In addition, phyB, in the presence of BR, inhibits and enhances the expression of the CPD gene in FR and R light, respectively. The R and FR responses in the presence of BR are mediated by nuclear and cytosol localized phyB, respectively, as examined by the nuclear and cytosol localized phyB.

Conclusion and Prospects: In crosstalk of light- and brassinosteroid-mediated control of *Arabidopsis* seedling growth, both FR light and R lights perceived by phyB have active functions but in an opposite manner. The phyB-mediated actions of FR and R light occur at cytosol and nucleus, respectively. Our observations may lead to revision of a major dogma in plant physiology. It is also well envisioned that cellular partitioning of a protein may be related to differential functions in plants.

Introduction

It is critical for photosynthetic plants to perceive and process their environmental light information properly. Phytochromes are one of the major photoreceptors in plants and control wide aspects of plant physiology by recognizing the light in red (R) and far-red (FR) spectra. Higher plants have two types of phytochromes; the photo-labile type I (phyA in *Arabidopsis*) and photo-stable type II (phyB-phyE in *Arabidopsis*) (Chen et al., 2004; Mathews, 2006). The type II phytochromes exhibit the classical interconversion between the two long-lived photoisomers, the R light-absorbing Pr (λmax; 660 nm) and the FR light-absorbing Pfr (λmax; 730 nm). Initially produced in the cell is Pr, which is then converted to Pfr upon receiving R light. Type II phytochromes have been considered, since its discovery, to function in plant responses to R light through formation of Pfr, the so-believed biologically active photoisomer. FR light can convert Pfr back to Pr. The Pr photoisomer has been largely believed biologically inactive (Chen et al., 2004), despite of a few reports that implicated the function of Pr (Lisculm and Hangarter, 1993). Interestingly, the Pr and Pfr photoisomers show distinctive cytosolic and nuclear portioning (Sakamoto and Nagatani, 1996, Chen et al., 2003). Here, we studied the crosstalk between light and brassinosteroids (BRs) mediated seedling growth, which led to the proposition that the cytosolic and nuclear photoisomers have different cellular functions.

Results and Discussion

Seedling development in higher plants is critically controlled by external light environment perceived by photoreceptors such as phytochromes but is also highly concerted with internal growth hormones including brassinosteroids (BRs) (Nemhauser et al., 2003). We were testing possible crosstalk between phyB, a type II

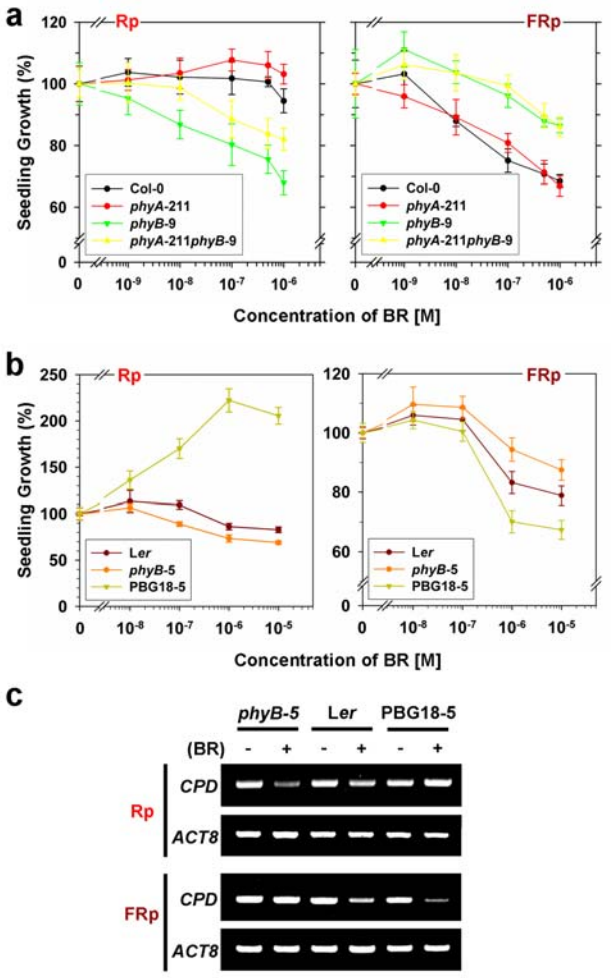


Figure 1. BR-responses of growth and gene expression of *Arabidopsis* seedlings in Rp and FRp. (a) BR-response of seedling growth of wild type (ecotype Col-0) and phytochrome mutants. *phyA*-211 and *phyB*-9 are null alleles of *Arabidopsis* phytochrome A and B genes, respectively. The seedlings were grown under various concentrations of epi-brassinolide (BR) with intermittent pulsed irradiation of R light (Rp) or intermittent pulsed irradiation of FR light (FRp) following each R light pulse (Supplementary Fig. 1). Seedling growth response was measured as the length of hypocotyls in the presence of BR relative to that in the absence of BR under the given light regime (mean ± s.d.; *n*=3, at least 50 seedlings per n). The actual growth data are given in Supplementary Fig. 2. (b) BR-response of seedling growth of wild type (ecotype *Ler*), *phyB*-5 null mutant, and a transgenic line overexpressing phyB-GFP fusion protein (PBG18-5). (c) BR-response of the *Arabidopsis* CPD gene expression in *phyB*-5, wild type (*Ler*), and PBG18-5 in Rp and FRp. The seedlings were grown in the absence (-) or presence (+) of 1 μM of BR. Total RNA extracted from approximately 500 seedlings per sample was subjected to RT-PCR followed by gel electrophoresis. An *Arabidopsis* actin gene (*ACT8*) was used as a normalization control.

phytochrome (Chen et al., 2004; Mathews, 2006), and BR in controlling Arabidopsis seedling growth (Chory and Li, 1997). For this test, we employed Arabidopsis mutants lacking phyA (*phyA*-211), phyB (*phyB*-9), both of phyA and phyB (*phyA*-211*phyB*-9) and their isogenic wild-type (Col-0). We compared their growth responses to exogenously supplemented BR under the intermittent R (Rp) and FR (FRp) light irradiation (Supplementary Fig. 1), the light regime that effectively leads to R and FR light responses, respectively (Shinomura et al., 2000). In Rp, exogenous *epi*-brassinolide (Hu et al., 2000), an active BR, led to a clear growth inhibition response in *phyB*-9 but this growth inhibition response was relieved in Col-0 seedlings (Fig. 1a and Supplementary Fig. 2). The BR response of seedling growth in FRp showed a striking

contrast to that in Rp; wild type seedlings became more sensitive to BR-mediated growth inhibition than *phyB*-9 null mutant seedlings (Fig. 1a and Supplementary Fig. 2). Thus, the roles of phyB in Rp and FRp were opposite; phyB enhanced and inhibited BR-responsive growth in Rp and in FRp, respectively. The single phyA-211 and the double *phyA*-211 *phyB*-9 mutants showed BR responses in a manner similar to Col-0 and to single phyB-9 mutant, respectively, in both Rp and FRp (Fig. 1a), indicating that the opposite effect on BR-responsive seedling growth observed in our experiment is mediated mostly through phyB, a type II phytochrome, but not by phyA, a type I phytochrome. We also observed the opposite effect of phyB, in Rp and FRp, on BR responses of seedling growth in another ecotype (*Ler*), utilizing the *phyB*-5 null mutation (Fig. 1b and Supplementary Fig. 3). This result reflects that the effect we observed was not ecotype-specific but phyB-specific character. The opposite effect of phyB on BR responses of seedling growth in Rp and FRp was much more evident in PBG18-5 transgenic line (ecotype *Ler*) that overproduces functional phyB as phyB-GFP fusion protein (Matsushita et al., 2003) (Fig. 1b and Supplementary Fig. 3). We further confirmed the opposite effect of phyB on BR responses in Rp and FRp by examining BR-regulated expression of the *Arabidopsis CPD/CYP90A1* gene in the *phyB*-5 null mutant, *Ler*, and PBG18-5. The *CPD/CYP90A1* gene encodes a BR biosynthesis enzyme and is feedback-regulated by exogenous BR (Tanaka et al., 2005). In our experiment, expression of this gene was also mostly down-regulated by exogenous BR. However, the degree of the feedback regulation showed a reciprocal trend in Rp and FRp (Fig. 1c); it was reduced in the order of the increasing (*phyB*-5, *Ler*, and PBG18-5) and decreasing (PBG18-5, *Ler*, and *phyB*-5) amounts of phyB in Rp and FRp, respectively.

The above result together clearly showed that phyB has an active function in modulating BR-responses of seedling growth and gene expression even in FRp, where phyB has long been believed to be physiologically inactive (Chen et al., 2004). Even further, the BR response through phyB was opposite in Rp and FRp. How are then the opposite roles of phyB in Rp and FRp brought about? The BR response of *Arabidopsis* seedlings in darkness (Dc) (Supplementary Fig. 4) was similar to that in FRp and thus was opposite to that in Rp (Fig. 1a and Supplementary Fig. 2). The distinctive feature of phyB is its differential nuclear and cytoplasmic partitioning in concomitant with photoisomerization; the newly synthesized Pr-phyB is mostly cytoplasmic in darkness and R light triggers translocation of the cytoplasmic phyB into the nucleus (Kircher et al., 1999, Nagy et al., 2001). We tested if the nucleo-cytoplasmic partitioning of phyB may lead to its opposite roles in Rp and FRp. For this, we employed transgenic plants expressing green fluorescent protein (GFP) fusion with wild-type phyB (PBG) and with mutant versions of PBG (Matsushita et al., 2003). PBG(G767/R) harbors amino acid transition of G767 to R, which renders the protein to lose the nuclear translocation ability. PBG(G767/R)-NLS is the constitutively nuclear localized version of PBG(G767/R) regardless of light regimes. These mutant phytochromes retain the same spectral and functional competence as PBG (Matsushita et al.,

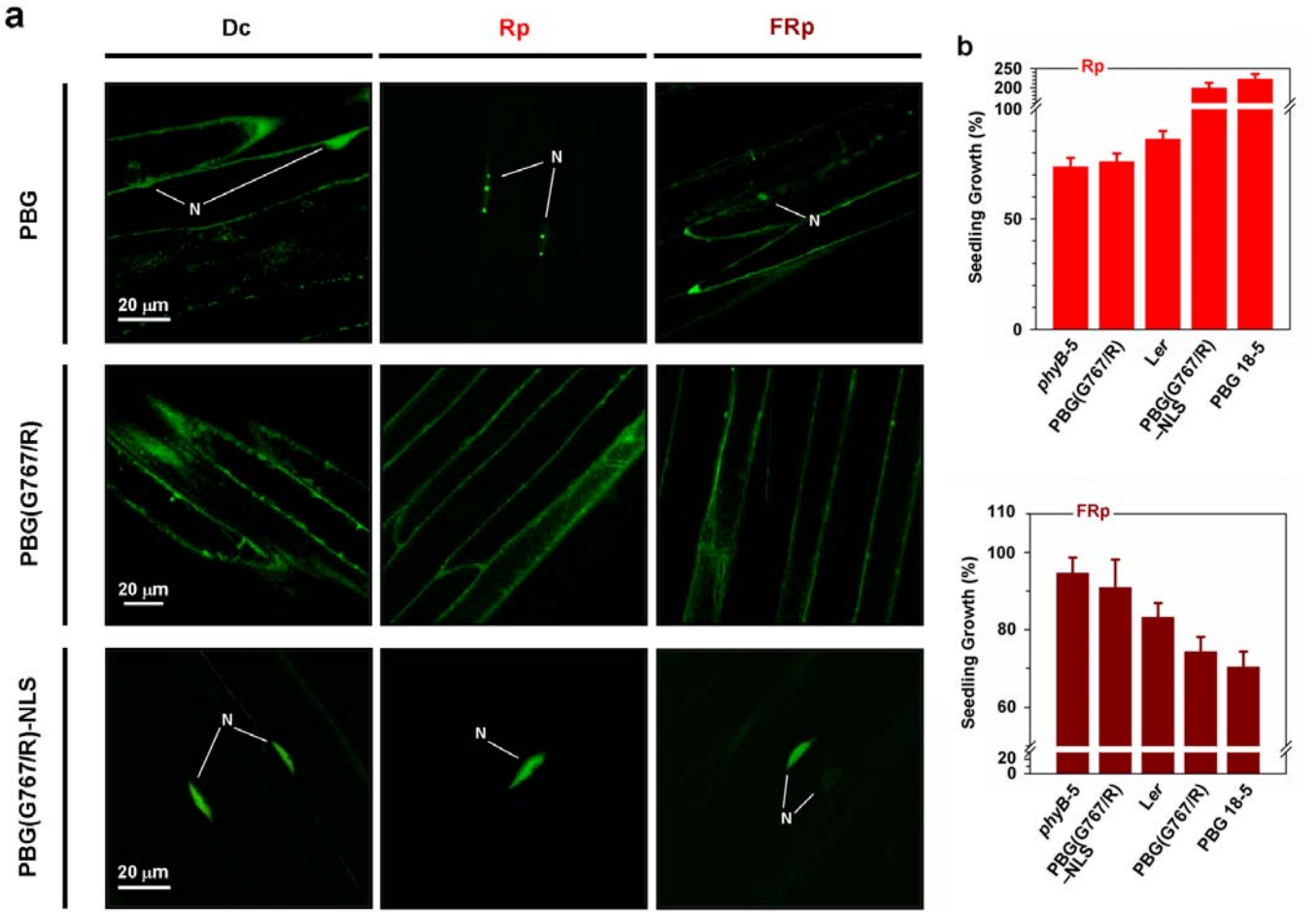


Figure 2. Comparison of BR-responses of nucleus- and cytoplasm-partitioned phyB. (a) Sub-cellular localization patterns of PBG18-5, PBG(G767/R) and PBG(G767/R)-NLS in hypocotyl cells of transgenic lines. The green fluorescent signals in the cortex and epidermal layers of 5-day-old seedlings grown under Rp, FRp, and Dc (Supplementary Fig. 1) were monitored by confocal microscopy. N, nucleus. (b) BR-response of *phyB*-5 null mutant, wild type (*Ler*), and PBG18-5, PBG(G767/R) and PBG(G767/R)-NLS transgenic seedlings in Rp and FRp. BR response of seedlings grown in media containing 1 μM of BR was noted as the length of hypocotyls relative to that in the absence of BR (mean ± s.d.; *n*=3, at least 50 seedlings per *n*). The actual data are given in Supplementary Fig. 4.

2003).

PBG was mainly detected in cytoplasm and in nucleus under Dc and Rp, respectively, in our experimental set-up. In FRp, where FR light was given after each R light irradiation, PBG became mostly cytoplasmic (Fig. 2a). In contrast, PBG(G767/R) and PBG(G767/R)-NLS was almost exclusively cytoplasmic and nuclear, respectively, in all the light regimes (Fig. 2a). We then compared the BR responses of seedling growth and the *CPD/CYP90A1* gene expression in these transgenic plants. In Rp, PBG(G767/R)-NLS seedlings showed a BR response similar to PBG seedlings (PBG18-5), but PBG(G767/R) seedlings showed a response similar to *phyB*-5 null mutant seedlings (Fig. 2b and Supplementary Fig. 5). The result indicates that in Rp the BR response was mediated through the nuclear localized phyB and that the cytoplasmic phyB has little role in this response. In a clear contrast, in FRp, PBG(G767/R) seedlings showed BR response in a degree similar to PBG18-5 seedlings but PBG(G767/R)-NLS seedlings showed a BR response similar to the phyB-5 null mutant seedlings (Fig. 2b and Supplementary Fig. 5). The result indicates that the cytoplasmic phyB in FRp is mediating the BR responses actively. A same trend is observed in the feedback regulation of the *CPD/CYP90A1* gene in these seedlings (Supplementary Fig. 6).

Conclusion and Prospects

Our findings show that the crosstalk actions of phyB and BR in seedling growth and gene expression critically depend on the cellular localization of phyB together with the light regime; the crosstalk actions occur oppositely in the nucleus and cytoplasm and the function of the nuclear and cytoplasmic phyB is manifested only in combination with R and FR light, respectively. Our findings thus note that FR light is not simply nullifying the R light effect but actively participates in seedling growth and gene expression in a manner opposite to R light in the phyB and BR crosstalk.

It would make much more sense that plants utilize the two isoforms rather than only one form, to effectively monitor the changing environmental light information and to incorporate the information into their developmental programs. There are now numerous cases where a protein is differentially partitioned in the cell depending on the signaling status. It is well envisioned that these differentially partitioned proteins may have differential functions in a cell. Here, we showed that the cytosolic and nuclear phyBs have opposite actions in seedling growth and gene expression in the seedling response to BR. A recent report (Wu and Spalding, 2007) that cryotochrome 1 shows separate nuclear and cytosolic functions in Arabidopsis seedling also support the notion. A spatially-differentiated function of a protein appears to be another layer of cellular regulation of a protein function.

Materials and Methods

Plant materials

phyA-211 is a null mutant allele of the *Arabidopsis thaliana PHYA* locus in Columbia-0 (Col-0) background (Reed et al., 1994). The *phyB-9* and *phyB-5* mutants are null alleles of the *PHYB* locus in Col-0 and Landsberg *erecta (Ler)* background, respectively (Reed et al, 1993). The PBG18-5, PBG(G767/R) and PBG(G767/R)-NLS lines in the *phyB-5* background were described previously (Matsushita et al., 2003).

Seedling growth media

Seedlings were grown on 0.8% agar plates containing 0.1 x Murashige-Skoog basal salt medium (Sigma-Aldrich Co. Ltd., Irvine, UK) without sucrose and with or without BR. The BR we used was *epi*-brassinolide and was obtained from Sigma-Aldrich Co. Ltd. The BR-containing media were prepared by adding appropriate amounts of stock solutions (10^{-2} M) in dimethyl sulfoxide (Fisher Scientific, Pittsburgh, PA, USA) to 25 ml of the media. Seeds, after sterilization and stratification, were sown on the agar plates and were then exposed to white light (200 μmol m^{-2} s^{-1}; F48T12/CW/VHO, Philips) for 24 hr to promote germination. After a further incubation for 15 min in far-red (FR) light (10 μmol m-2 s-1) at 22°C, the seeds were placed under various light conditions for 5 days before measuring seedling growth.

Light conditions and seedling growth measurement

Seedling growth measurement was conducted by measuring hypocotyl length of seedlings. For measurement of BR response of hypocotyl growth under various light conditions, seedlings were subjected to intermittent irradiations of red (R) light (30 μmol m^{-2} s^{-1} for 15 min each) at intervals of 105 min darkness, or intermittent irradiations of R light (30 μmol m^{-2} s^{-1} for 15 min each) followed by FR light (10 μmol m^{-2} s^{-1} for 15 min each) at intervals of 90 min darkness. The light regime that effectively leads to R and FR light responses, respectively (Shinomura et al., 2000). The R and FR lights were obtained from R- and FR-light emitting diodes (Good feeling Co Ltd., Seongnam, Republic of Korea) after filtering through a 3mm thick R-acrylic plate (Shinokolite A 102; Mitsubishi Rayon, Tokyo, Japan) and a FR-acrylic plate (Deraglass 102; Asahikasei Co Ltd., Tokyo, Japan), respectively. Hypocotyl length was determined by using Hewlett Packard ScanJet 3770 digital scanner (Hewlett Packard, USA) and Scion image software (Beta 4.0.2, Scion Corporation, Rockville, MD, USA) and was calculated as mean ± SD. Each experiment was performed with at least 50 seedlings.

RNA preparation and RT-PCR

Total cellular RNA was isolated from seedlings using the RNeasy Plant Miniprep kit (QIAGEN Sciences, Germantown, MD, USA). The first-strand cDNA was then synthesized from 2.5 μg of total RNA using Avian Myeloblastosis Virus reverse transcriptase (Promega, Madison, WI, USA) and was subjected to PCR. The oligo nucleotides 5'-TCCTCCTCCTCTCTTCCATCGCCG-3' and 5'-ACGGCGCTTCACGAAGATCGGGTA-3' were used for detection of the *CPD/CYP90A1* transcript. The oligo nucleotides 5'-TCCGAGTTTGAAGAGGCTACAAAC-3' and 5'-AATCAGATGTGGATCTCTAAGGCA-3' were used for amplification of *ACT8* transcript. The PCR program was composed of the following profile: initial denaturation for 3 min at 94°C; 5 cycles of 94°C (30 sec), 55°C (30 sec), 72°C (30 sec), 25 cycles of 94°C (30 sec), 52°C (30 sec), 72°C (1 min), and final elongation for 7 min at 72°C. The PCR products were resolved on 0.7% (w/v) agarose gel.

Microscopic observations

The specimens were observed with an inverted laser scan confocal microscope (Fluoview FV1000; Olympus, Japan) equipped with ×10 and ×60 objectives. The laser scan images were obtained with a combination of 488 nm laser excitation and with a spectral filter for 500-600 nm emission.

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