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A Small GTPase Activator Protein Interacts with Cytoplasmic Phytochromes in Regulating Root Development*[S]

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Phytochromes enable plants to sense light information and regulate developmental responses. Phytochromes interact with partner proteins to transmit light signals to downstream components for plant development. PIRF1 (phytochrome-interacting ROP guanine-nucleotide exchange factor (RopGEF 1)) functions as a light-signaling switch regulating root development through the activation of ROPs (Rho-like GTPase of plant) in the cytoplasm. *In vitro* pulldown and yeast two-hybrid assays confirmed the interaction between PIRF1 and phytochromes. PIRF1 interacted with the N-terminal domain of phytochromes through its conserved PRONE (plant-specific ROP nucleotide exchanger) region. PIRF1 also interacted with ROPs and activated them in a phytochrome-dependent manner. The Pr form of phytochrome A enhanced the RopGEF activity of PIRF1, whereas the Pfr form inhibited it. A bimolecular fluorescence complementation analysis demonstrated that PIRF1 was localized in the cytoplasm and bound to the phytochromes in darkness but not in light. PIRF1 loss of function mutants (*pirf1*) of *Arabidopsis thaliana* showed a longer root phenotype in the dark. In addition, both PIRF1 overexpression mutants (*PIRF1-OX*) and phytochrome-null mutants (*phyA-211* and *phyB-9*) showed retarded root elongation and irregular root hair formation, suggesting that PIRF1 is a negative regulator of phytochrome-mediated primary root development. We propose that phytochrome and ROP signaling are interconnected through PIRF1 in regulating the root growth and development in *Arabidopsis*.

Light is the one of various environmental factors affecting plant root growth and development (1). Light-sensing photoreceptors such as phytochromes and cryptochromes regulate root growth and development, including lateral root growth (2), gravitropism (3), and root elongation (4). The root hair formation is particularly sensitive to light signals perceived by phytochromes (1). In phytochrome-mediated root developmental events such as lateral root growth and gravitropism, positive photomorphogenic factor HY5 plays a crucial regulatory role in *Arabidopsis* (5, 6). Phytochromes synthesized as inactive Pr forms in the dark change conformations into active far-red light-absorbing Pfr forms by red light. This photochromism of phytochromes enables plants to modulate red/far-red light signals to regulate developmental responses (7–10).

Current interest in phytochrome-mediated root development concerns root hair elongation, primary root formation, and photo- and gravitropisms (3, 4, 11–17). To discover the role of the phytochrome in roots, we studied the possible involvement of the phytochrome-interacting protein. In this regard, RopGEF (Rho of plants guanine nucleotide exchange factor) identified previously as a phytochrome-interacting protein (18) possibly participates in root development. ROPs play a signaling role in diverse developmental processes and regulate primary root elongation, lateral root formation, and root hair polarity in response to various environmental factors (19–25). Relevant to our study, RopGEF proteins play a critical role in ROP signaling through their ability to activate ROPs involved in the control processes of plant growth and development (22, 26–28). A previous study suggested the possible involvement of ROPs in the phytochrome signaling pathway, as the production of GTP by an NDPK2 enzyme was specifically activated by the Pfr form of phytochrome A (29).

In the present study, we suggest that RopGEF11 (PIRF1; phytochrome interacting RopGEF 1) activated an ROP in a phytochrome-dependent manner for root development in *Arabidopsis*. Interestingly, we found that cytoplasmic PIRF1 interacted with the N-terminal region of phytochromes A and B localized in the cytoplasm. Based on studies with PIRF1 knockout and overexpression mutant plants, we explore the functional role of the cytoplasmic phytochromes and PIRF1 in regulating root development through activation of an ROP.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions—The *Arabidopsis* T-DNA insertion mutant (*pirf1*, Salk_126725) of PIRF1 and the phytochrome-null mutants (*phyA-211* and *phyB-9*) were obtained from the Salk Institute and *Arabidopsis* Stock Center,

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Methods, Table S1, Figs. S1–S11, and an additional reference.

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respectively. A homozygote of *pirf1* was selected by genomic PCR, and the inhibition of *PIRF1* gene expression in *pirf1* was confirmed by reverse transcription-PCR (RT-PCR)[3] analysis using the gene-specific primers (see supplemental Table S1). The *PIRF1* overexpression mutant (*PIRF1-OX*) was generated by transformation of the *35S-myc-PIRF1* construct into wild type *Arabidopsis* (Col-0). The overexpression of *myc-PIRF1* in *PIRF1-OX* was confirmed by RT-PCR and Western blot analysis with monoclonal anti-myc antibodies (Millipore, Billerica, MA) (data not shown). For seedling growth and light response experiments, *Arabidopsis* seeds were surface-sterilized and sown on plates containing one-half Murashige & Skoog medium, pH 5.7 and 0.8% Phyto agar (Duchefa Biochemie, Haarlem, The Netherlands). The plates were positioned vertically to allow root growth along the gel surface. Images of plant samples were captured using a Nikon D-70s digital camera, and the primary root lengths were measured using Image-Tool software (University of Texas Health Science Center, San Antonio, TX).

Light Conditions—Seedlings were irradiated with either continuous far-red (FRc; 730 nm, 23 μmol m^{-2} s^{-1}) or red (Rc; 660 nm, 12 μmol m^{-2} s^{-1}) light. Monochromatic red or far-red light was generated by light-emitting diodes (GFLE-102R, GoodFeeling, Gyeonggi-Do, Korea). Light intensities were measured using a Quantum-Photo Radiometer HD9021 (Delta OHM SRL, Selvazzano, Italy).

Yeast Two-hybrid Assay—For yeast two-hybrid analyses of PIRF1 and phytochromes, PIRF1 protein was fused to the GAL4 DNA binding domain. The N terminus (phyA-N or phyB-N) and C terminus (phyA-C or phyB-C) of phytochromes A (phyA) and B (phyB) were subcloned into the pGADT7 vector to express truncated proteins fused with the GAL4-activation domain. Yeast two hybrid assays were then performed according to the manufacturer's recommended conditions (Matchmaker Two-hybrid System, Clontech, Mountain View, CA).

Co-immunoprecipitation Assay—The myc-tagged *PIRF1-OX* plants were grown in darkness for 4 days at 22 °C after germination. The seedlings were then harvested and homogenized with an extraction buffer (70 mM Tris-HCl, pH 8.3, 35% ethylene glycol, 98 mM $(NH_4)_2SO_4$, 7 mM EDTA, 14 mM sodium metabisulfite) containing protease inhibitor mixture (Roche Applied Science). Monoclonal anti-myc antibodies (Millipore) were incubated with the crude protein extracts at 4 °C overnight followed by incubation with protein G-agarose (Millipore) at 4 °C for 3 h. The beads were then washed six times with washing buffer (50 mM Tris-HCl, pH 7.4, 150 mM $NaCl_2$, and 1% Nonidet P-40) and resuspended in 2× SDS-PAGE sample buffer. Proteins were separated on a 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane (Invitrogen). Anti-myc (4A6, Millipore), anti-phyA (mAA for *Arabidopsis* phyA (30)) and anti-phyB (mBA for *Arabidopsis* phyB (30)) monoclonal antibodies were then used for detecting PIRF1 and two phytochromes, respectively. Horseradish peroxidase-conjugated anti-mouse IgG was used as the secondary antibody, and the signals were detected by chemiluminescence using an ECL reaction (GE Healthcare).

Histochemical GUS Assay—For histochemical β-glucuronidase (GUS) assays for *PIRF1*, ~0.9 kb of the promoter region of the *PIRF1* gene was amplified by PCR and cloned into the pBI101 binary vector (Clontech). The resulting construct was then transformed into wild type *Arabidopsis* plants (Col-0), and transformants were selected on one-half mass spectrometry media containing 30 μg/ml kanamycin. Histochemical staining and microscopic analyses were performed as described previously (31).

GDP/GTP Exchange Assays—The GDP/GTP exchange activity of PIRF1 with ROP proteins was measured by the nitrocellulose filter binding method at room temperature essentially as described previously by Zheng *et al.* (32). Briefly, for the measurement of GDP binding, purified recombinant maltose binding protein tagging ROPs (MBP-ROPs) were rendered nucleotide-free by incubation for 5 min in a loading buffer containing 2 mM EDTA and 3 mM [^{3}H]GDP, followed by the addition of 5 mM $MgCl_2$ and a further incubation for 20 min. Purified GST-PIRF1 was equilibrated for 15 min in an exchange buffer containing 1 mM GTP and then added to the ROP-containing solution. The reaction was then continued for the indicated periods. For the binding of [^{35}S]GTP (guanosine 5′-[γ-thio]-triphosphate), 3 μM unlabeled GDP instead of [^{3}H]GDP was used in the loading buffer and 5 μM [^{35}S]GTP instead of unlabeled GTP in the exchange assay buffer. Bound and free nucleotides were separated by filtration with nitrocellulose.

Transient Co-expression and Image Analysis of Arabidopsis Protoplast Cells—To confirm the subcellular co-localization and interaction between PIRF1 and phytochromes, we adopted a BiFC analysis using *Arabidopsis* protoplast cells. The cDNAs of phytochrome A and B were individually subcloned into BiFC vector (33) containing the N-terminal region of yellow fluorescent protein (YFP) and designated *NE-PHYA* and *NE-PHYB*, respectively. The cDNA of *PIRF1* was also cloned into the BiFC vector containing the C-terminal region of YFP to yield *CE-PIRF1*. Protoplast preparations and transient transformations were performed according to the methods described by Yoo *et al.* (34). Protoplasts were isolated by incubating 3-week-old *Arabidopsis* leaves in enzyme solution containing cellulase and macerozyme, and the transformation of protoplasts was achieved by incubating plasmid DNA and protoplast in polyethylene glycol-calcium solution (40% PEG 4000, 0.2 M mannitol, 100 mM $CaCl_2$). Protoplasts co-transformed with *CE-PIRF1* and *NE-PHYA* and *NE-PHYB* were incubated in darkness for 14 h, and the fluorescence emissions were measured. To examine the effects of light quality on the co-localization of PIRF1 and phytochromes, the transformed protoplasts were incubated in darkness for 14 h and kept in darkness or exposed subsequently to red or far-red light for 5 min and kept a further 30 min in darkness. Confocal imaging was then carried out using a LSM 510 META laser scanning microscope (Carl Zeiss, Thornwood, NY). Image analysis was carried out with LSC Image 5 Examination software (Carl Zeiss).

RESULTS

PIRF1 Is a Member of the RopGEF Family—In our previous study, we used the co-immunoprecipitation matrix-assisted laser desorption ionization time-of-flight/mass spectrometry

[3] The abbreviations used are: RT-PCR, reverse transcription-PCR; YFP, yellow fluorescent protein; GST, glutathione *S*-transferase; GUS, β-glucuronidase.

method to screen holophytochrome-interacting proteins and identified a 66-kDa protein in *Arabidopsis* (18). Genomic analysis further revealed the presence of an ortholog of this protein on chromosome 1. Based on genomic and database analyses, the 66-kDa protein and its ortholog were identified as the RopGEF family proteins, RopGEF11 (PIRF1) and RopGEF13 (PIRF2), respectively. The characteristic conserved PRONE domain of RopGEF family members (27, 28, 35) was found in these two proteins (see supplemental Fig. S1*A*). PIRF1 consisted of a 1620-bp open reading frame encoding 539 amino acids for a 58.6-kDa protein. A sequence alignment of PIRF1 and -2 shows 66.3% overall homology, which rises up to 80.9% within the conserved PRONE domain regions. PIRF1 and PIRF2 are phylogenetically distinguishable from other RopGEF members (see supplemental Fig. S1*B*).

PIRF1 Interacts with N-terminal Region of Phytochromes—To confirm the interaction between PIRF1 and phytochromes, we generated a recombinant GST-PIRF1 protein and performed *in vitro* pulldown assays with several recombinant oat phyAs. Full-length oat phytochrome A interacted with GST-PIRF1. In addition the N-terminal region (AN) of phyA was sufficient for the interaction with PIRF1 (see supplemental Fig. S2). The specific interactions between phytochromes and PIRF1 were further confirmed by yeast two-hybrid assays, showing that both phytochromes A and B interacted with PIRF1 through their N-terminal but not through C-terminal domains (Fig. 1*A* and supplemental Fig. S3). To further establish the interaction between PIRF1 and phytochromes *in vivo*, we generated myc-tagged PIRF1-overexpressing transgenic plants (*PIRF1-OX*). The myc-PIRF1 proteins were immunoprecipitated by myc antibodies from the crude protein extracts of *PIRF1-OX* seedlings. Western blotting analysis revealed that PIRF1 interacted with phyA and phyB in *PIRF1-OX* seedlings (Fig. 1, *B* and *C*).

Arabidopsis—RopGEF family proteins contain a conserved central PRONE domain with the variable N- and C-terminal regions. Their GEF activity toward ROPs reside in the PRONE domain (27). The variable regions of RopGEFs, however, are responsible for the regulation of PRONE function of RopGEF proteins (36). To better understand the structural requirement for the PIRF1 and phytochrome interactions, we generated various truncated GST-PIRF1 proteins and performed *in vitro* pulldown assays (Fig. 2). Phytochrome A bound with full length and PRONE domain-containing (NP and PD) recombinant PIRF1 proteins. However, neither the N terminus (NE) nor C terminus (CE) alone interacted with phytochrome A. These results indicate that the N-terminal domain of the phytochrome A and the PRONE domain of PIRF1 provide docking sites for PIRF1-phytochrome interactions.

PIRF1 Interacts with and Activates ROPs—In plants, ROPs are involved in diverse developmental processes through their activation by RopGEFs (22, 26). We examined the interaction and activation of PIRF1 over ROPs using *in vitro* pulldown assay and GDP/GTP exchange assay to verify the activity of PIRF1 as a functional RopGEF. Three typical ROPs (ROP2, ROP6, and ROP8) were selected from 11 ROPs for *in vitro* pulldown assay. Results revealed that PIRF1 directly interacted with three ROPs (Fig. 3*A*). We then tested the GEF activity of PIRF1

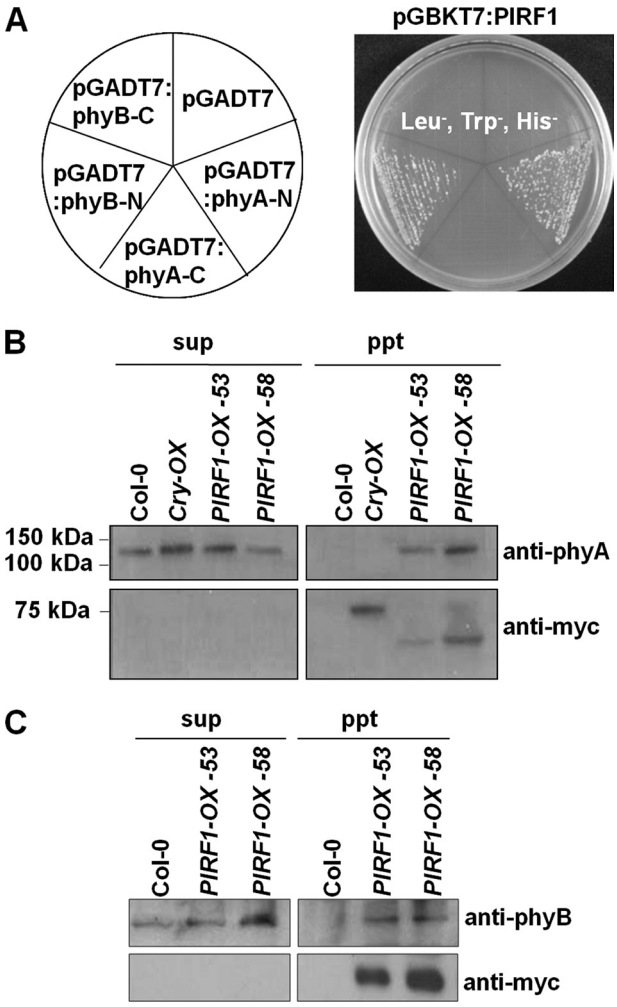

FIGURE 1. **PIRF1 interacts with phytochromes.** *A*, yeast two-hybrid assay of the interaction between PIRF1 and *Arabidopsis* phytochromes. The triple-dropout plate indicates a positive association between the bait (PIRF1) and prey (*Arabidopsis* PHYA or *Arabidopsis* PHYB) on a SD/Leu$^-$/Trp$^-$/His$^-$ plate. Both N-terminal regions of phytochrome A (pGADT7:phyA-N; 1–616 aa) and phytochrome B (pGADT7:phyB-N; 1–649 aa) were found to interact with PIRF1 (pGBKT7:PIRF1; 1–539 aa) but not C-terminal regions of phytochromes (pGADT7:phyA-C; 617–1122 aa, pGADT7:phyB-C; 650–1172 aa). *B* and *C*, co-immunoprecipitation of PIRF1 and phytochromes from *myc-PIRF1* overexpression transgenic plants. Crude extracts obtained from dark grown *PIRF1-OX* seedlings were precipitated using monoclonal anti-myc antibodies, followed by Western blotting with anti-phyA (*B*), anti-phyB (*C*), and anti-myc antibodies. *Cry-OX* is a myc-tagged cryptochrome 1 overexpression mutant used as a control. *sup*, supernatants; *ppt*, precipitates.

with one of the ROPs tested. The dissociation of [^{3}H]GDP from MBP-ROP8 was accelerated with the addition of increasing concentrations of PIRF1 (Fig. 3*B*). The rates of both [^{3}H]GDP dissociation and [^{35}S]GTP binding to MBP-ROP8 were markedly enhanced by PIRF1 (Fig. 3*C* and supplemental Fig. S4). These results suggest that PIRF1 plays a functional role as a RopGEF protein in plants.

RopGEF Activity of PIRF1 Was Enhanced by a Pr-phytochrome A—ROP signaling is modulated by an interacting RopGEF proteins through regulation of their GEF activities. In tomato, pollen-specific receptor kinases LePRK1 and -2 interact with a tomato RopGEF homolog, LeKPP, and regulate its GEF activity (36, 37). Because PIRF1 interacts with phytochromes and ROPs, we tested whether phytochromes have any

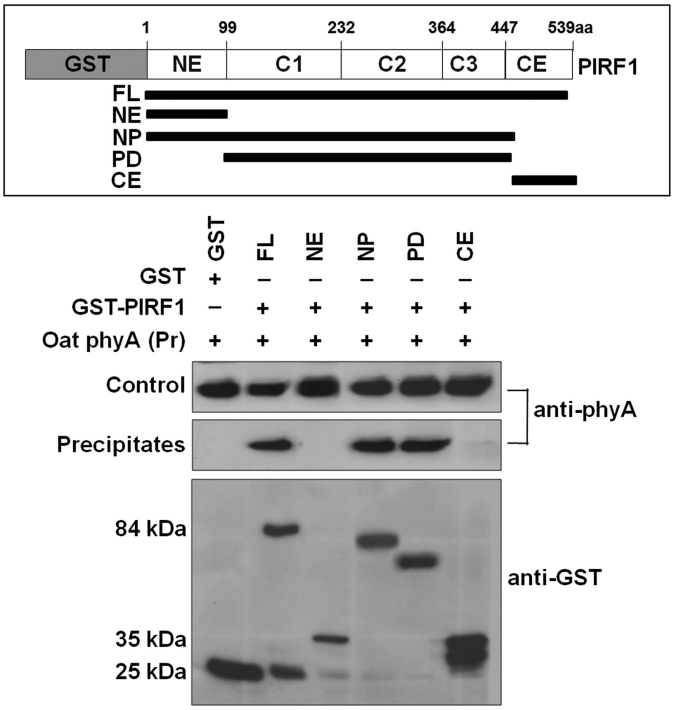

FIGURE 2. **PRONE domain is required for PIRF1-phytochrome interactions.** *In vitro* binding assays were performed with phytochrome A and truncated PIRF1s. Recombinant oat phyA was incubated with the truncated GST-PIRF1s (*NE*, N-terminal element; *NP*, N-terminal element and PRONE domain; *PD*, PRONE domain; *CE*, C-terminal element) or GST. The resulting reaction complexes were pulled down with glutathione-Sepharose 4B beads. The binding of phyA to GST-PIRF1 was then detected by Western blotting using anti-phyA and anti-GST antibodies. *FL*, full-length PIRF1; *aa*, amino acids.

effect on PIRF1 activity. Surprisingly, PIRF1 activity was enhanced highly by the Pr form of phytochrome A, whereas the Pfr form decreased the activity (Fig. 4). These results suggest that the Pr form of phytochromes modulates ROP signaling by controlling the GEF activity of PIRF1 in *Arabidopsis*.

PIRF1 Interacts with Phytochromes in Cytoplasm—Another surprising finding in our study was that the interaction between PIRF1 and phytochromes occurred in the cytoplasm. The subcellular translocation of signaling molecules including photoreceptors plays a crucial role in the regulation of gene expression and protein degradation in plant photomorphogenesis (38). Light induces the translocation of phytochromes from the cytoplasm to the nucleus, where gene expression is directly modulated to control many developmental processes in plants (8, 9). Thus, many phytochrome signaling proteins such as PIF3 and PIF-like (PIL) are localized and interact with phytochromes in the nucleus (39, 40).

To explore the functional role of PIRF1, subcellular localization was analyzed using green fluorescent protein-tagged PIRF1 protein in *Arabidopsis* protoplast cells. PIRF1 was found to be localized in the cytoplasm of protoplast cells regardless of light exposure (see supplemental Fig. S5). To assess the possibility of the cytoplasmic interaction between PIRF1 and phytochromes at different light conditions, we used the BiFC method by co-transformation of PIRF1 (*CE-PIRF1*) and either phyA (*NE-PHYA*) or phyB (*NE-PHYB*). The interaction between PIRF1 and phytochromes in the transformed cells was observed in the dark (Fig. 5*A*) but absent in red light exposure (Fig. 5*B*),

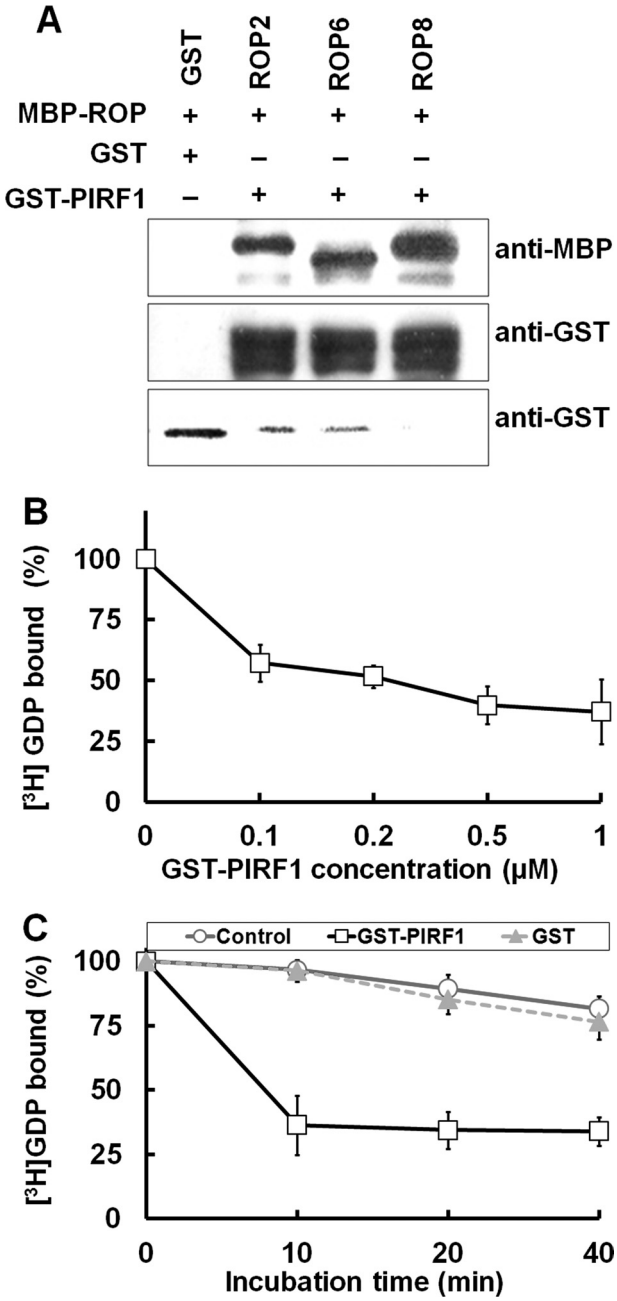

FIGURE 3. **PIRF1 interacts with ROP proteins and accelerates a GDP/GTP exchange.** *A*, *in vitro* pulldown assay of PIRF1 and ROPs. GST-PIRF1 was incubated with several MBP-fused ROP proteins (*MBP-ROP*). ROP2, ROP6, and ROP8 were found to interact with PIRF1. *B* and *C*, the guanine nucleotide exchange activity of PIRF1. The release of [^{3}H]GDP from MBP-ROP8 was accelerated with the addition of increasing concentrations of PIRF1 (*B*). MBP-ROP8 was incubated with different amounts of PIRF1 for 30 min, and [^{3}H]GDP release from MBP-ROP8 was then measured. Time course analysis of [^{3}H]GDP release from MBP-ROP8 (*C*). The [^{3}H]GDP release from MBP-ROP8 was measured in the absence (○) or presence (▲) of GST as a control and in the presence of GST-PIRF1 (□).

whereas the interaction between PIF3 and phytochromes in the nucleus was activated by the same light treatment (see supplemental Fig. S6). To confirm that PIRF1 interacts with phytochrome in cytoplasm, we monitored the changes of fluorescent cells as well as fluorescence transition from the cytoplasm to the nucleus by light illuminating the protoplast cells. Protoplasts were co-transformed with the *NE-PHYA* and *CE-*

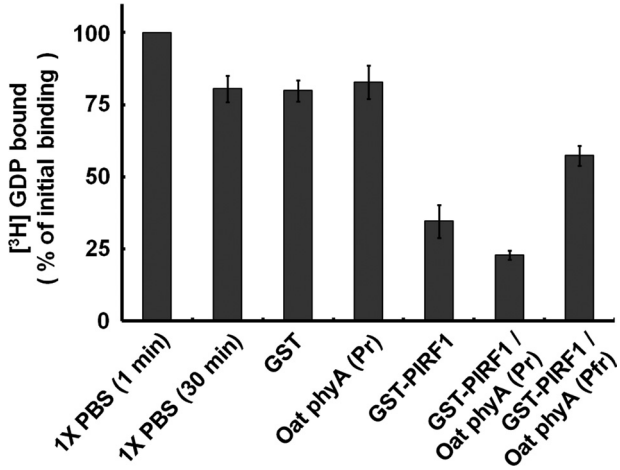

FIGURE 4. **The GEF activity of PIRF1 is regulated by phytochromes.** The recombinant oat phytochrome A (both the Pr and Pfr forms) was incubated with GST-PIRF1 and MBP-ROP8 in the reaction mixture, and the [^{3}H]GDP release from MBP-ROP8 was then analyzed. The GEF activity of PIRF1 was enhanced by the Pr form of phyA but inhibited by the Pfr form of phyA. Phosphate-buffered saline (*PBS*), GST, and oat phyA (Pr) alone were used as a control to test their nonspecific activities. *Error bars* indicate standard deviations from three independent measurements.

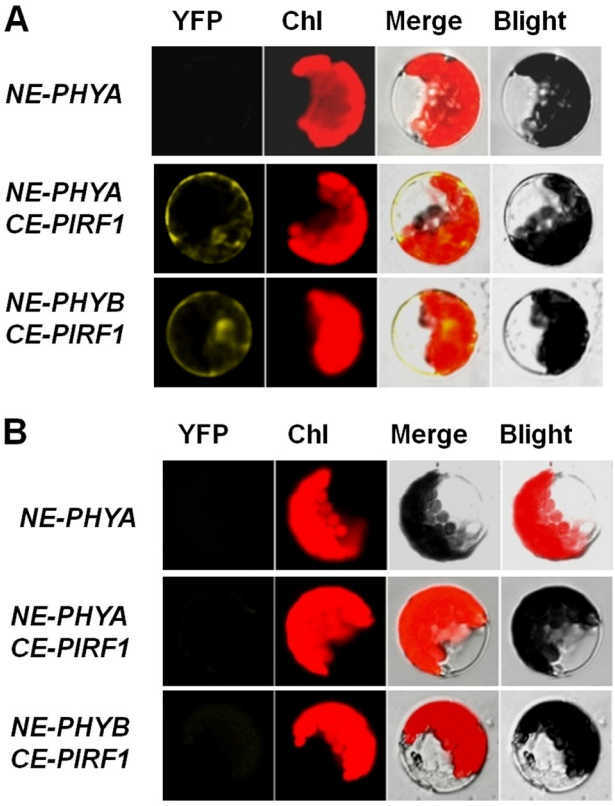

FIGURE 5. **PIRF1 interacts with phytochromes in the cytoplasm.** The YFP-tagged phytochrome (either *NE-PHYA* or *NE-PHYB*) and PIRF1 (*CE-PIRF1*) were cotransformed into *Arabidopsis* protoplasts, which were then incubated in darkness for 14 h. After dark incubation, the cells were kept in darkness (*A*), or exposed to red light for 5 min and kept a further 30 min in darkness (*B*) to evaluate the interaction and co-localization of these proteins. The fluorescent images revealed that PIRF1 and phytochromes co-localize and interact in the cytoplasm. *YFP*, yellow fluorescent protein; *Chl*, chlorophyll autofluorescence; *YFP/Chl*, overlap of YFP (*yellow*) and Chl (*red*); *Bright*, bright field images.

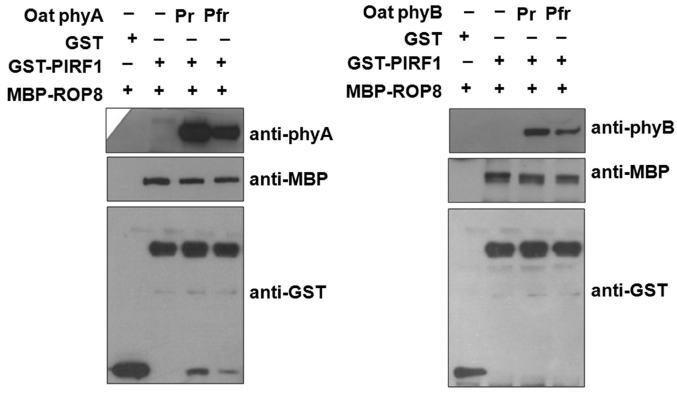

FIGURE 6. **The complex of PIRF1 and ROP binds dominantly to the Pr form of phytochromes *in vitro*.** Recombinant oat phytochrome A and B (both the Pr and Pfr forms) were incubated with GST-PIRF1 and MBP-ROP8 and pulled down with glutathione-Sepharose 4B beads. The association of phyA and phyB to the complex of GST-PIRF1 and MBP-ROP8 was then detected by immunoblotting using anti-phyA (*left*) or anti-phyB (*right*), anti-MBP, and anti-GST antibodies.

PIRF1, *CE-PIF3*, or *CE-PHYA*. As expected, the dimerized phytochromes, NE-phyA and CE-phyA, were translocated from the cytoplasm to the nucleus with a red light pulse where the interaction was still maintained. In addition, translocated NE-phyA interacted with CE-PIF3 in the nucleus. However, NE-phyA and CE-PIRF1 interaction in the cytoplasm was markedly decreased by red light pulses (see supplemental Fig. S7). Therefore, PIRF1 is localized in the cytoplasm, where it probably binds the Pr form of phytochrome in the dark, whereas this association is abrogated by the red light induced translocation of phytochromes into the nucleus. Our results suggest that PIRF1 as a phytochrome partner protein may play a role in the cytoplasm.

Pr Specifically Binds the PIRF1-ROP Complex—We showed that the GEF activity of PIRF1 was differentially regulated by Pr and Pfr forms of phytochrome, suggesting that the interaction between PIRF1 and phytochrome was Pr-specific, whereas an *in vitro* pulldown assay showed no significant differences in interaction between PIRF1 and Pr or Pfr form of phytochromes (see supplemental Fig. S8). However, phytochrome Pr form interacted preferentially with PIRF1 when it was complexed with ROP8 (Fig. 6). This result explains why the Pr form of phytochrome is more specific in activating the GEF activity of PIRF1 (Fig. 4).

PIRF1 Expression Is Dominant in Roots and Flowers—A quantitative mRNA analysis showed that *PIRF1* expression was stronger in roots and flowers than in other tissues examined, whereas *PIRF2* was weakly expressed only in mature flowers (Fig. 7*A*). These distinct expression patterns of *PIRF1* and *PIRF2* suggest that the former, rather than the latter, likely plays a crucial role in vegetative development of *Arabidopsis*. In addition, histochemical analysis of *PIRF1* promoter-glucuronidase transgenic plants (*PIRF1-GUS*) revealed that *PIRF1* was expressed strongly in actively elongating regions of roots (also in pollen) (Fig. 7*B*). The dominant expression patterns of *PIRF1* in roots and pollen closely matched those of *PHYB* expression in *PHYB-GUS* transgenic mutants (31), suggesting that PIRF1 together with phyB plays a role in the development of these tissues.

PIRF1 Negatively Regulates Root Development—Having isolated and characterized the phytochrome-interacting protein PIRF1, we searched for its function in plant photomorphogen-

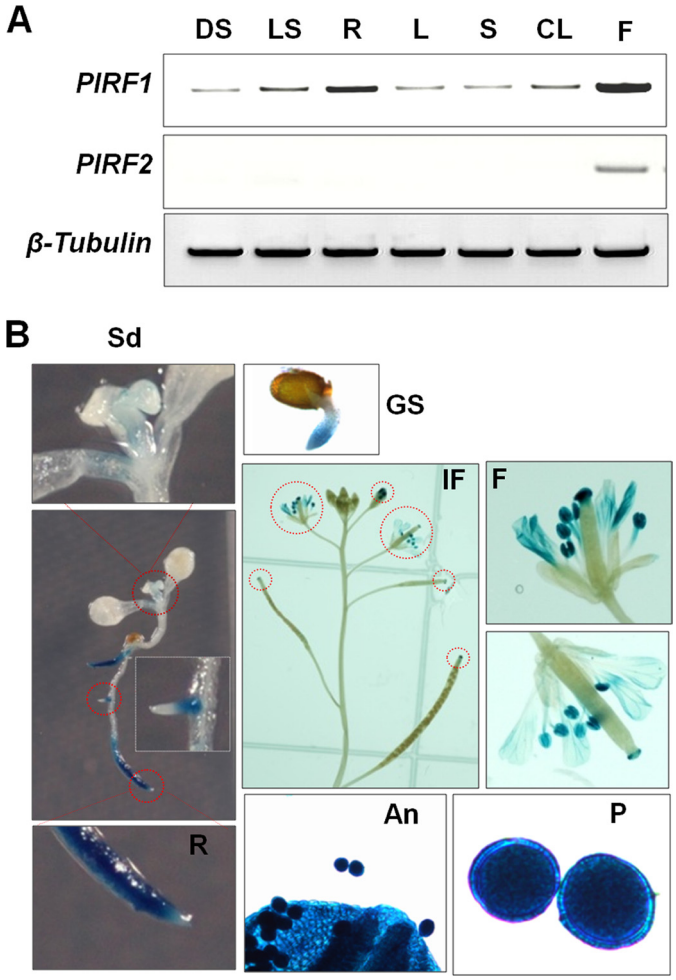

FIGURE 7. **PIRF1 is expressed dominantly in roots and flowers.** *A*, expression profiles of *PIRF1* and *PIRF2*. *PIRF1* and *PIRF2* transcript levels in different tissues of *Arabidopsis* were analyzed by RT-PCR. Total RNA was isolated from seedlings and different tissues of 3-week-old plants. *DS*, dark grown seedlings; *LS*, light grown seedlings; *R*, roots; *L*, rosette leaves; *S*, stems; *CL*, cauline leaves; *F*, flowers. *B*, histochemical analysis of *PIRF1* expression in *PIRF1-GUS* transgenic plants. *PIRF1-GUS* transgenic plants were germinated and grown on the one-half Murashige & Skoog media containing 2% sucrose for 2 weeks. Then, they were transplanted to soil and grown in a greenhouse. The results showed that GUS expression is detected in the radicle of germinating seeds (*GS*), the roots (*R*) of 3-day-old seedlings (*Sd*) grown under white light, inflorescences from adult plants (*IF*), flowers (*F*), anthers (*An*), and pollen (*P*).

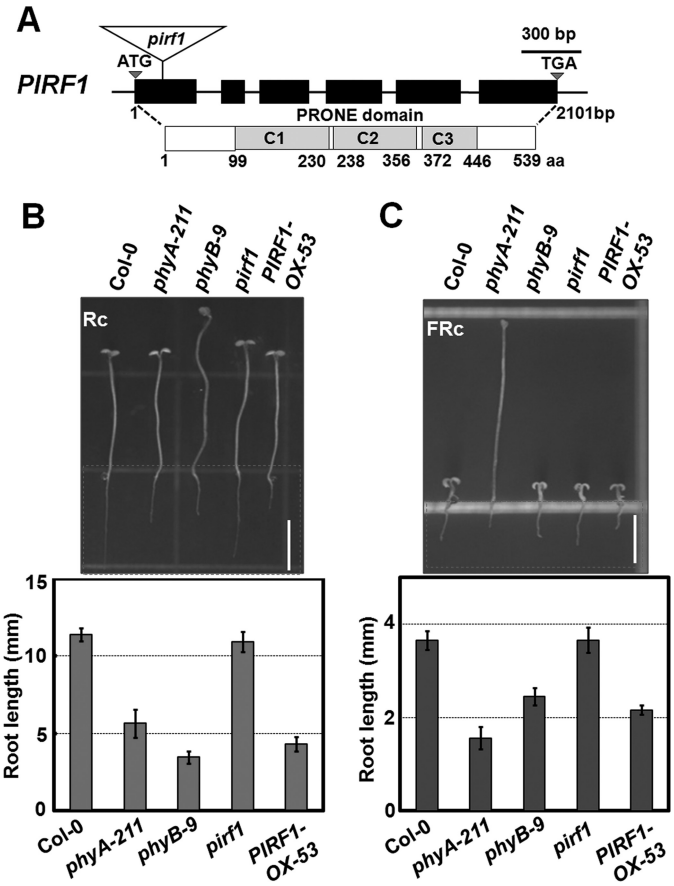

FIGURE 8. **PIRF1 is involved in the primary root development.** *A*, a schematic depiction of the exon/intron structures of the *PIRF1* gene. The T-DNA insertion position is indicated. *C1–C3* represents the subdomains of the conserved PRONE domain in the RopGEF family. *B* and *C*, primary root growth in the *PIRF1-OX* mutant seedlings is retarded under continuous red light (*B*) and far-red light (*C*). The light responses of wild-type plants, PIRF1 mutants (*pirf1* and *PIRF1-OX*), and phytochrome-null mutants (*phyA-211* and *phyB-9*) in terms of root development were analyzed by measuring the primary root length of 1-week-old seedlings grown under continuous red light (*B*) or continuous far-red light (*C*). Both PIRF1 overexpression mutants and phytochrome null mutants showed retarded primary root growth under red and far-red light. *Error bars* represent the mean ± S.E. ($n \geq 30$) of five independent experiment. *Bar*, 5 mm.

esis. Light signaling (3, 6) as well as ROP signaling (19, 21) plays important roles in root development, including primary root elongation and root hair formation. First, we attempted to establish the phenotypic functional relationship between PIRF1 and phytochromes by generating T-DNA insertion knock-out (*pirf1*) (Salk_126725) (Fig. 8*A*) and overexpression mutants (*PIRF1-OX*) of *A. thaliana*. We analyzed the de-etiolation responses of these mutant seedlings, including hypocotyl growth, cotyledon opening, and greening under different light conditions. We could not observe any distinct difference in phenotype in *pirf1* during early seedling development, but *PIRF1-OX* displayed inhibited root growth. We then looked at root phenotypes in these mutant plants under different light conditions. Primary roots of seedling in wild type plants were markedly elongated under white light, but the growth was retarded in the dark (see supplemental Fig. S9). However, primary root elongation was markedly lower in *PIRF1-OX* seedlings under continuous white, red, and far-red lights than wild type and *pirf1* seedlings (see supplemental Fig. S9). The elongation of primary roots in the phytochrome null mutants, *phyA-211* and *phyB-9*, also was inhibited under both continuous red (Rc) or far-red (FRc) light conditions suggesting the involvement of both phytochromes A and B (Fig. 8, *B* and *C*, and supplemental Fig. S9). In addition, *pirf1* and phytochrome B null mutants showed elongated root growth in the dark. Inhibitions of primary root growth by PIRF1 overexpression, and elongation of root in the *pirf1* and phytochrome B null mutants in the dark, strongly suggest PIRF1 as a negative regulator by modulating its ROPs activity. This suggestion is consistent with the primary root elongation in transgenic ROP overexpressors (41). The lack of a distinct root growth phenotype in the *pirf1* mutant is probably due to a functional redundancy among the RopGEF family members, similar to that among ROP families (42). On the other hand, the primary root lengths in *PIRF1-OX* and *phy* mutants were shorter than those of the wild type plants at fluence rates >0.1 mol^{-2} s^{-1}, which suggest that PIRF1 func-

tions under high red or far-red light intensities (see supplemental Fig. S10).

Phytochrome null mutants showed defective root hairs in seedlings under light conditions (1). We also observed similar deficiency in root hair formation in phytochrome null mutants (*phyA-211* and *phyB-9*) under continuous red or far-red light. A phenotype analysis of *PIRF1-OX* plants revealed that the overexpression of *PIRF1* repressed a root hair formation (see supplemental Fig. S11). The similarity of root phenotypes in phytochrome null and *PIRF1-OX* mutants suggests that PIRF1 and phytochromes are on the same signaling pathway for the root development. In particular, we observed that the polarity of the root hairs also was disturbed markedly in the *PIRF1-OX* plants compared with the wild type, as was the case with phytochrome B null mutant. Relevantly, the disruption of root tip polarity also has been observed in transgenic ROP plants (19, 21, 41). Our results suggest that phytochromes and ROPs are required for root hair development and that PIRF1 serves as a signal connector between phytochrome and ROPs.

DISCUSSION

PIRF1 Is a Cytoplasmic Partner Protein of Pr-phytochrome—A number of potential signaling components that are specific to an individual phytochrome response appear to mostly interact with the C-terminal region of the phytochromes (7, 8, 38, 40, 43). However, the phytochrome N-terminal region has a regulatory function implicating that the N-terminal region could be a signaling domain for the photomorphogenesis in higher plants (9, 10, 44, 45). For instance, PAPP2C was found to interact with an N-terminal motif of the phytochromes in *Arabidopsis* and regulates the de-etiolation responses of seedlings by regulating the dephosphorylation of PIF3 (46). In the present study, we also demonstrate that PIRF1 functions as a partner protein through its interaction with the N-terminal regions of phytochromes (Fig. 1 and supplemental Figs. S2 and S3). Unlike most other phytochrome-interacting proteins, PIRF1 interacted specifically with an N-terminal domain of both phyA and phyB.

Phytochromes reside predominantly in the cytoplasm in dark grown seedlings and are translocated into the nucleus upon light irradiation to trigger diverse light responses (47, 48). Nuclear localization of phyA and phyB is a pivotal event to elicit the signaling pathway leading to photomorphogenesis in *Arabidopsis* (47–49). It also appears that cytoplasmic phytochromes play a role in phototropism and root development of plant seedlings even when the nuclear translocation of phyA or B was blocked (50, 51). Our findings show that PIRF1 localized and interacted with Pr-phytochromes in the cytoplasm in the dark, and it remained there even after Pr was converted to Pfr upon light exposure (Fig. 5 and supplemental Figs. S5 and S7).

PIRF1-Pr Complex Acts as a "Signal Switch" of ROP Signaling in Root Development—In most photomorphogenic events in higher plants, the Pfr form of phytochrome is the functionally active form (or "switch on" form) with the Pr form being silent ("switch off" form). Occasionally, the Pr form has been suggested as an active form (52, 53). Shinomura *et al.* (54) suggested the possibility that a Pr form (re-formed from the Pfr form) can play a role in the red/far-red pulse effects on the inhibition of hypocotyl elongation in seedling. Correll and Kiss (4) also suggested that root-localized phytochromes are able to regulate the root growth response to light through the inactive red-absorbing form (Pr) of phytochrome. Kang *et al.* (55) showed the induction of a small G protein and a brassinosteroid C2 hydroxylase (a cytochrome P450) in the dark grown *Arabidopsis* seedlings, implicating the possible role of the Pr form. Recent studies also have suggested that the cytosolic Pr form of phytochromes has a biological function during plant development (16, 50, 51). In addition, our results showed that the RopGEF activity of PIRF1 was enhanced by the Pr form of phytochromes but inhibited by the Pfr form (Fig. 4). Also, the Pr form of phytochromes interacted predominantly with the complexes of PIRF1 and ROP8 *in vitro* (Fig. 6), whereas no preference between Pr and Pfr forms was observed in the interaction of PIRF1 with phytochromes when ROP is excluded (see supplemental Fig. S8). Our results suggest that the Pr-phytochrome is nonsilent in the cytoplasm.

Phytochromes have been known to present abundantly in roots and root tip through spectrophotometric and immunocytochemical studies (56). Somer and Quail (31) also demonstrated that phytochrome genes were expressed highly in root through promoter analysis using GUS-tagging phytochromes. Various studies also showed that phytochromes are involved in various root development including root hair development (11), lateral root formation (5, 6), and tropism responses (3, 13). It was particularly interesting that light was able to penetrate through the interior of the stem and was conducted toward the roots (50). Although phyB and red light play a role in root development, no nuclear phyB-green fluorescent protein was detected in root cells that were >1 cm below the soil line (16). These findings suggest that light signals can directly propagate to the root system and trigger phytochrome activation through vascular conductance, but phyB activation for nuclear movement was not achieved via weak light signals. Salisbury *et al.* (16) proposed the possibility that cytosolic-localized Pr plays a role in controlling several responses during root development. This possibility was coincided with our results that Pr form of phytochrome might be involved in root development through interaction with PIRF1 and ROP in cytoplasm.

The previous reports (3, 4, 13, 16) and the results obtained from the present study strongly indicate that the phytochromes are able to detect light directly and play an important role in plant root development. Our results showed that the impairment of phytochrome signaling results in abnormalities in both root growth and root hair formation (Fig. 8 and supplemental Fig. S11). This observation is consistent with the report that phytochromes mediate the root development through regulating the expression of auxin response genes and auxin efflux genes (16); for example, *hy5* mutation affects root developments by regulating specific auxin-responsive genes (5, 6). Tian *et al.* (57) also showed that phyB regulates AUX/IAA activity by directly interacting with SHY2/IAA3. These observations are consistent with the phytochrome-mediated modulation of intercellular signalings, such as auxin transport and distribution, in controlling root development (16, 53). In addition, involvement of ROPs in auxin signaling is well known (26, 35). ROPs stimulate auxin-responsive gene expression (58) and

functions as novel regulators for the proteolytic degradation of AUX/IAA, negative transcription regulator of auxin response gene expression (59). Also, our *PIRF1-OX* plants displayed abnormal root phenotypes similar to those of transgenic constitutively active ROP (CA-ROP) overexpressors (19, 21, 41), suggesting that root development is negatively regulated by ROPs. To probe the roles of PIRF1 in auxin-responsive gene expression, we examined the expression of several auxin response genes (*IAA1*, *IAA3*, and *ARF*) and efflux genes (*PIN3*, *PIN4*, and *PIN7*) in *pirf1* and *PIRF1-OX* mutants. However, no variation in the gene expression of them was observed in those mutants (data not shown), suggesting that PIRF1 was not directly involved in the regulation of auxin-responsive gene expression.

As shown in our results (Fig. 8 and supplemental Fig. S10), phytochrome null mutants displayed severely retarded growth under red and far-red light. Overexpression of cry1 significantly increased the root elongation, whereas the knock-out mutant also showed reduced growth (60). Blue light signal through cry1 for root growth is transmitted via the auxin-signaling pathway. Phototropin1 also enhanced root growth efficiently and increased plant size and maturity (61). Therefore, root growth is the result of various light-signaling responses through different photoreceptors. In the present study, we found that the Pr-phytochromes interact with PIRF1 to modulate RopGEF activity in the cytoplasm, which possibly influences the root growth through the regulation by ROPs. These results establish the possible link between light and ROP signals in root growth and development. Thus, the Pr-PIRF1 pair appears to serve as a signaling switch for root growth inhibition. However, this switch is not the only switch for root growth and development because the knock-out mutants of phytochromes and *pirf1* exhibited only minor effects in the dark. Nonetheless, the retardation of root growth in the PIRF1 overexpression mutant under light conditions strongly implicates a possible connection between phytochromes and ROPs via PIRF1 during root growth and development. Further studies are warranted to establish the mechanism of how phytochromes/light, PIRF1, and ROPs play a signaling role in root growth and development.

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