Journal of Plant Biology (2021) 64:181–191
https://doi.org/10.1007/s12374-021-09302-9

RESEARCH ARTICLE

Generation and Characterization of a Specific Polyclonal Antibody against *Arabidopsis thaliana* Phytochrome-Interacting Factor 3

Da-Min Choi[1] · Jae-Yong Cho[1] · Woe-Yeon Kim[2] · Yun-Jeong Han[3] · Jeong-Il Kim[1,3]

Received: 30 December 2020 / Revised: 20 January 2021 / Accepted: 21 January 2021 / Published online: 10 February 2021

Abstract

Phytochrome-interacting factors (PIFs) are a basic helix-loop-helix family of transcriptional regulators that maintain skotomorphogenesis and suppress photomorphogenesis. PIFs are regulated by plant photoreceptors, especially phytochromes. In general, PIFs physically interact with phytochromes, and this interaction induces PIF's phosphorylation and subsequent degradation, contributing to the initiation of photomorphogenic development. Among the eight members of PIF (PIF1 to PIF8) reported in *Arabidopsis thaliana*, PIF3 is the first discovered member and plays central roles in de-etiolation and chlorophyll biosynthesis. More recently, PIF3 has been also reported to regulate hormone signaling and cold tolerance in plants. Although PIF3 protein shows dynamic behaviors in plants, its study has been limited due to the lack of an authentic PIF3 antibody. In this study, we produced polyclonal antibodies using inclusion bodies and characterized the PIF3 antibody based on specificity and sensitivity. In addition, we investigated PIF3 phosphorylation and degradation during phytochrome-mediated light signaling in plants. Furthermore, we successfully performed in vitro protein–protein interaction and co-immunoprecipitation assays between phytochrome B (phyB) and PIF3 using the antibody. Therefore, we obtained an authentic PIF3 antibody that could be used as a valuable tool to study the multi-faceted functions of PIF3.

Keywords Phytochrome · Phytochrome-interacting factors · PIF3 · Polyclonal antibody · Protein degradation

Introduction

Higher plants respond flexibly to their surroundings as sessile organisms. Among environmental cues, light is an essential element, not only for photosynthesis to produce the necessary energy as autotrophs, but also for plant growth and development by responding to fluctuating environments (Jing and Lin 2020). The monitoring of light in plants is mediated by various photoreceptors, including phytochromes (Legris et al. 2019). Phytochromes are red (R) and far-red (FR) photoreceptors that regulate various plant photoresponses, such as germination, de-etiolation, shade avoidance, leaf senescence, and flowering (Tripathi et al. 2019). Phytochromes are known to function as a molecular switch with physiologically active FR light-absorbing (Pfr) and inactive R light-absorbing (Pr) forms (Li et al. 2011). Upon absorbing light, the Pr-to-Pfr photoactivation induces a highly regulated signaling network for plant growth and development in response to light environments, which includes the translocation of phytochromes into the nucleus and interaction of phytochromes with a wide array of signaling partners (Hoang et al. 2019; Legris et al. 2019).

There are tens of known signaling partners that physically interact with phytochromes, suggesting that phytochromes regulate plant light signaling via protein–protein interactions (Bae and Choi 2008). Among them, phytochrome-interacting factors (PIFs) are suggested as central players in phytochrome-mediated light signaling networks (Leivar and Quail 2011). PIFs belong to bHLH (basic helix-loop-helix) transcription factors with conserved active phytochrome-binding motifs in the N-terminal domain, and eight PIFs have been identified in *Arabidopsis thaliana* (Pham et al.

✉ Yun-Jeong Han
hanyj0716@gmail.com

✉ Jeong-Il Kim
kimji@jnu.ac.kr

[1] Department of Integrative Food, Bioscience and Biotechnology, Chonnam National University, Gwangju 61186, Korea

[2] Department of Applied Life Chemistry, Gyeongsang National University, Jinju 52828, Korea

[3] Kumho Life Science Laboratory, Chonnam National University, Gwangju 61186, Korea

2018a; Favero 2020; Oh et al. 2020). They usually promote seedling skotomorphogenesis, i.e., etiolated growth in the dark, such as long hypocotyls, closed cotyledons, and no chlorophyll biosynthesis. In contrast, PIFs repress seedling photomorphogenesis, including seed germination and de-etiolation, through the regulated expression of more than a thousand genes (Shin et al. 2009; Pham et al. 2018b). As another important signaling component for phytochrome-mediated light signaling, a member of bZIP (basic leucine zipper) transcription factor family, elongated hypocotyl 5 (HY5) is a master transcription factor that promotes photomorphogenesis downstream to photoreceptors (Lee et al. 2007; Gangappa and Botto 2016). Therefore, the principal regulatory mechanism of phytochromes for light signaling might be the transcriptional regulation of photoresponsive genes via the inactivation of negative transcriptional factors such as PIFs and via the accumulation of positive transcriptional factors such as HY5 (Tripathi et al. 2019; Jing and Lin 2020).

Among PIFs, phytochrome-interacting factor 3 (PIF3) is the founding member that negatively regulates phytochrome-mediated light signaling (Ni et al. 1998; Kim et al. 2003). The interaction of PIF3 with photoactivated phytochromes leads to its phosphorylation and subsequent degradation via the ubiquitin/26S proteasome pathway (Al-Sady et al. 2006). More recently, phytochromes are reported to function as protein kinases that can directly phosphorylate PIF3 (Shin et al. 2016). In addition, PIF3 phosphorylation can be occurred due to the influence of other kinases (Ni et al. 2017). Collectively, a regulatory model for phytochrome-mediated photomorphogenesis has been suggested. Upon light exposure, photoactivated phytochromes move to the nucleus, where they physically interact with PIFs and induce their phosphorylation and protein degradation, contributing to the initiation of the photomorphogenic development of plants (Hoang et al. 2019; Favero 2020). Therefore, the phytochrome-mediated removal of PIFs, the negative regulators of photomorphogenesis, might play a critical role in plant light signaling.

Although PIFs were originally discovered in the phytochrome-mediated light signaling pathways, they may also have functions in integrating multiple signaling pathways (Paik et al. 2017). In particular, PIF3 functions in chlorophyll biosynthesis, the regulation of diurnal hypocotyl elongation, and the modulation of cotyledon opening during de-etiolation (Shin et al. 2009; Soy et al. 2016; Dong et al. 2019). In addition, PIF3 affects plant hormone signaling, including ethylene, auxin, gibberellin, and abscisic acid, and also regulates cold tolerance by regulating the expression of C-repeat binding factors (Bours et al. 2015; Li et al. 2016; Yu and Huang 2017; Jiang et al. 2020; Liang et al. 2020). Furthermore, PIF3 is involved in regulating the circadian response and protecting seedlings from reactive oxygen species (Chen et al. 2013; Soy et al. 2016). Therefore, the important function of PIF3 is becoming increasingly apparent.

Although there is such increasing data on the importance of PIF3 in plant growth and development being available, its study has been limited due to the lack of an authentic PIF3 antibody. In particular, PIF3 protein shows dynamic behaviors in plants, so temporal and spatial analyses of the PIF3 protein are required. Epitope tagging could be used to detect PIF3 in plants, but these methods require a considerable amount of time to generate transgenic plants and the behaviors of the tagged-PIF3 might not reflect the exact intrinsic function. Therefore, in this study, a polyclonal antibody with a high affinity to PIF3 was produced in rabbits through immunization to the electroeluted recombinant protein using inclusion bodies. The antibody specifically interacted with PIF3 but not with the seven other PIFs in Arabidopsis. With this antibody, light-dependent degradation and accumulation behaviors of PIF3 could be successfully analyzed in plants, and the elongated growth phenotype of phytochrome B (phyB)-deficient Arabidopsis could be explained by a higher PIF3 level at night than that in the control plant. Therefore, the PIF3 antibody obtained in this study could be helpful to determine the multiple functions of PIF3 in plant growth and development in the future.

Results and Discussion

Production of a Specific Polyclonal Antibody against PIF3

Previously, Arabidopsis GIGANTEA antibody (α-GI) was successfully produced with electroeluted proteins using inclusion bodies (Khaleda et al. 2017). Although the protein is denatured, this method provides large amounts of antigens with high purity, possibly inducing effective immune responses for antibody production. Thus, PIF3 was expressed as inclusion bodies (Supplementary Fig. S1), and used for polyclonal antibody production in rabbits after electroelution (hereafter, α-PIF3). Then, the specificity of the α-PIF3 antibody was investigated using different PIFs. For this, eight PIFs identified in Arabidopsis have been prepared using *Escherichia coli* protein expression systems (Supplementary Fig. S2 and Table S1). As PIFs are transcriptional factors, they are usually expressed as inclusion bodies, which makes it difficult to purify the recombinant proteins. To overcome this challenge, a two affinity-tag system has been developed and used for the purification of PIFs, in which GST- and strep-tags are fused to N- and C-termini, respectively (Jeong et al. 2016; Shin et al. 2016). With this system, recombinant proteins of six PIFs (PIF1, PIF3, PIF4, PIF6, PIF7, and PIF8) were expressed successfully and purified

by streptavidin affinity chromatography (see the SDS-PAGE gel in Fig. 1). However, the other two PIFs (PIF2 and PIF5) were not expressed in this system, so a cold-inducible promoter system (*pCold TF* vector) was used for their expression, including PIF3. Finally, all eight PIF proteins were successfully purified and used to test the specificity of the produced antibody. The results showed that the antibody interacted specifically only with PIF3 but not with the other seven PIFs (Fig. 1).

Next, endogenous PIF3 in plants was detected using the produced antibody with different dilutions. For this, dark-grown seedlings were used for the extraction of total proteins, because PIF3 is degraded in light in a phytochrome-dependent manner (Al-Sady et al. 2006). In this experiment, Col-0 (wild-type Arabidopsis), *pif3* (PIF3-deficient Col-0), and PIF3:eGFP (Col-0 expressing eGFP-fused PIF3) were included. As expected, an endogenous PIF3 band (524 aa; ~ 57.6 kDa) was detected in Col-0 but not in the *pif3* plant (Fig. 2a). In the PIF3:eGFP plant, endogenous PIF3 and eGFP-fused PIF3 (763 aa; ~ 83.8 kDa) bands were both detected. Interestingly, this result suggests that the endogenous expression level of PIF3 is higher than that in the transgenic plant with cassava vein mosaic virus (*CsVMV*) promoter. Thus, the expression level of PIF3 in transgenic plants with constitutive promoters, such as *35S* and *CsVMV*, will be lower than that in wild-type plants, which may be useful for interpreting the results of transgenic *pif3* plants expressing *PIF3*. Moreover, these experiments were conducted with different dilutions of the purified α-PIF3 antibody, and results showed that the 1:10,000 dilution was sufficient to detect endogenous PIF3 in Col-0 (Fig. 2a). These results indicate that the antibody produced in this study had high sensitivity to detect the antigen. Considering background noise in western blots, a dilution of 1:3000 was used for further analyses.

Among the eight PIFs, four (PIF1, PIF3, PIF4, and PIF5) play major roles in plant growth and development (Leivar et al. 2008; Shin et al. 2009; Pham et al. 2018a). Thus, immunoblotting was performed using the total extracts from

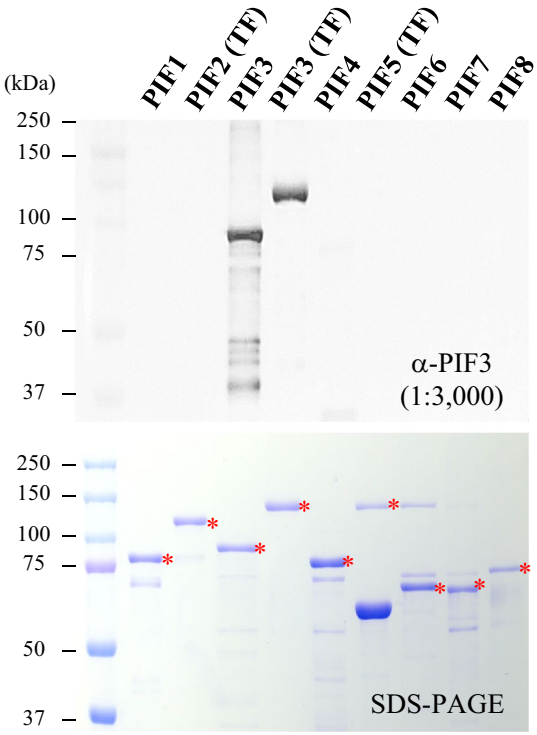

Fig. 1 Specificity test of the α-PIF3 antibody using recombinant proteins of eight PIFs in *Arabidopsis thaliana*. Six PIFs (PIF1, PIF3, PIF4, PIF6, PIF7, and PIF8) were expressed and purified as GST/strep-tagged proteins, and three PIFs (PIF2, PIF3, and PIF5) as TF/2B8/strep-tagged proteins (labeled as TF in parenthesis). It is notable that two purified PIF3 proteins with either GST/strep-tag or TF/2B8/strep-tag were used in this analysis. Approximately 1 µg of each purified recombinant protein was run on 10% SDS-PAGE and western blotting was performed with the purified α-PIF3 antibody generated in this study (1:3000 dilution). Asterisks (*) in red indicate protein bands of the corresponding PIFs

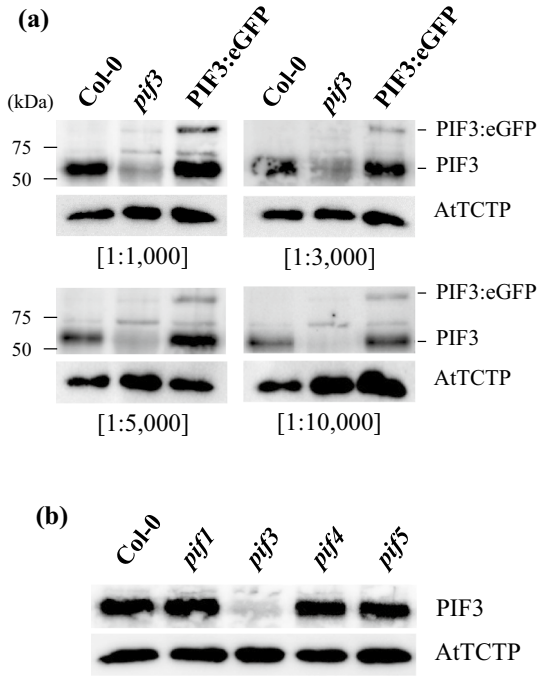

Fig. 2 Sensitivity and specificity tests of the α-PIF3 antibody in plants. **a** Western blots showing the sensitivity of α-PIF3 using 3.5-day-old dark-grown seedlings. Col-0, wild-type Arabidopsis; *pif3*, PIF3-deficient Arabidopsis; PIF3:eGFP, transgenic Col-0 expressing eGFP-fused PIF3. 80 µg of plant extract was run on 10% SDS-PAGE, and western blotting was performed using α-PIF3 with 1:1000, 1:3000, 1:5000, and 1:10,000 dilutions. *A. thaliana* translationally controlled tumor protein (AtTCTP; At3g16640) was used as the loading control. **b** Western blot showing the specificity of α-PIF3 using different *pif* mutants. 80 µg of plant extracts obtained from 3.5-day-old dark-grown seedlings of four *pif* mutants (*pif1*, *pif3*, *pif4*, and *pif5*) was used for western blotting with α-PIF3 (1:3000). AtTCTP was used as the loading control

pif1, *pif3*, *pif4*, and *pif5* plants with the α-PIF3 antibody, and the result confirmed in vivo specificity toward PIF3 (Fig. 2b). Collectively, an authentic α-PIF3 antibody with high specificity and sensitivity was successfully produced in the present study.

Phytochrome-Induced Degradation of PIF3 in Plants

PIF3 is phosphorylated and degraded in a phytochrome-dependent manner (Al-Sady et al. 2006; Shin et al. 2016). There are five phytochromes (phyA to phyE) in Arabidopsis, and it is known that phyA regulates FR light signaling, while phyB to phyE regulate R light signaling (Mathews 2010). Accordingly, PIF3 is degraded under FR light conditions in a phyA-dependent manner, and degradation under R light conditions is mainly mediated by phyB. To test this, immunoblotting was undertaken with plant extracts from L*er* (wild-type Arabidopsis), *phyA-201* (phyA-deficient L*er*), *phyAB* (i.e., *phyA-201phyB-5*; phyA- and phyB-deficient L*er*), and *phyABCDE* (all five phytochrome-deficient L*er*). In the L*er* plant, PIF3 was detected in the dark, but degraded under R, FR, and WL conditions (Fig. 3). In contrast, degradation of PIF3 in the *phyABCDE* plant was not observed. In the *phyA-201* plant, PIF3 was degraded under R and WL conditions but not under the FR condition, confirming that phyA is necessary for FR-mediated PIF3 degradation. On the other hand, PIF3 in the *phyAB* plant was degraded under the WL condition but not under the FR condition, suggesting the role of phyB in R-mediated PIF3 degradation. However, PIF3 was partially degraded in the *phyAB* plant under the R condition, which might be due to the presence of phyC–phyE that can also mediate R-light signaling (Franklin et al. 2003; Adam et al. 2013).

To further investigate phytochrome-mediated PIF3 degradation in plants, PIF3 degradation was analyzed in a time-dependent manner. In these experiments, A-OX (transgenic *phyA-201* expressing wild-type phyA) and B-OX (transgenic *phyB-5* expressing wild-type phyB) were included. FR-dependent PIF3 degradation was observed in L*er* and A-OX, but not in *phyA-201* and *phyABCDE* (Fig. 4a). When the degradation rates were estimated from immunoblots, PIF3 degradation was faster in A-OX than in L*er* (see the graph in Fig. 4a). This result indicates that the expression level of phyA in transgenic plants is higher than the endogenous phyA level, which is consistent with the observed shorter hypocotyls of A-OX in previous studies (Jeong et al. 2016; Shin et al. 2016). Under R conditions, PIF3 degradation was observed in L*er* and B-OX, but not in *phyABCDE* (Fig. 4b). In the case of *phyB-5* plant, PIF3 degradation was observed with a much slower rate, which is also explained by the presence of phyC–phyE. In addition, shifted PIF3 bands were observed during the degradation, indicating the occurrence of the phosphorylated forms (marked as PIF3-P). Overall,

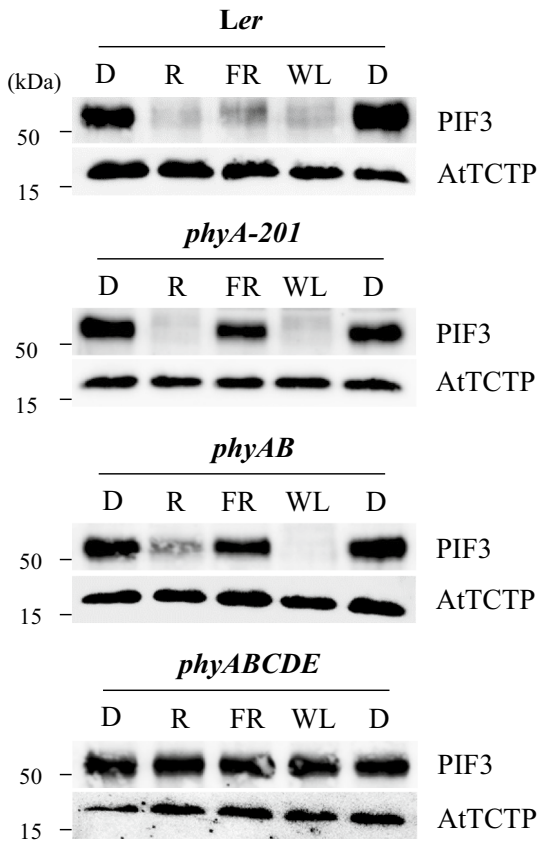

Fig. 3 Analysis of the light-induced degradation of PIF3 using α-PIF3 in plants. L*er*, wild-type Arabidopsis; *phyA-201*, phyA-deficient Arabidopsis; *phyAB*, phyA- and phyB-deficient Arabidopsis (*phyA-201phyB-5*); *phyABCDE*, all five phytochrome (phyA to phyE)-deficient Arabidopsis. Total proteins were extracted from 3.5-day-old dark-grown seedlings (D), or after irradiation with red (R, 10 μmol m^{-2} s^{-1}), far-red (FR, 5 μmol m^{-2} s^{-1}) or white light (WL, 100 μmol m^{-2} s^{-1}) for 30 min. 80 μg of extracted protein was used for western blotting with α-PIF3 (1:3000). AtTCTP was used as the loading control

the present study demonstrates that FR- and R-dependent PIF3 degradation is mediated by phyA and phyB–phyE, respectively.

PIF3 Accumulation in the Dark for Elongated Growth in phyB-Deficient Arabidopsis

Among phytochrome-deficient Arabidopsis, the *phyB* plant shows the most dramatic differences in growing phenotype, i.e., constitutive shade avoidance responses (Casal 2013; Martinez-Garcia et al. 2014). Under a canopy habitat, plants rapidly elongate their stems in search of light at the expense of leaf growth and reproductive development (Franklin and Whitelam 2005). Among these shade avoidance responses, the stimulation of elongation is remarkably rapid, usually causing dramatically accelerated flowering. Thus, the *phyB* plants show elongated phenotypes with early flowering.

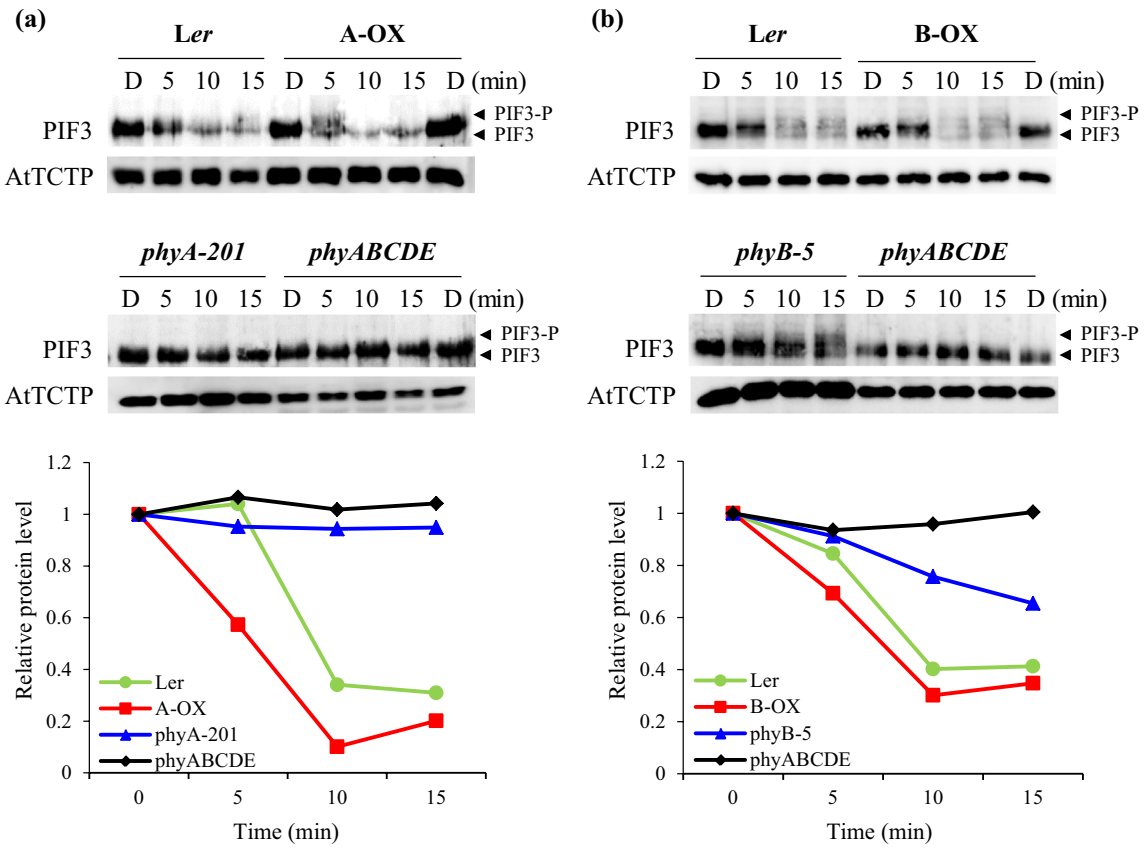

Fig. 4 Phytochrome-mediated PIF3 degradation and phosphorylation in plants. **a** FR-dependent degradation of PIF3. A-OX, transgenic *phyA-201* expressing wild-type phyA. **b** R-dependent degradation of PIF3. *phyB-5*, phyB-deficient Arabidopsis; B-OX, transgenic *phyB-5* expressing wild-type phyB. 3.5-day-old dark-grown seedlings (D) were irradiated with FR (5 μmol m^{-2} s^{-1}) or R (10 μmol m^{-2} s^{-1}) light for 5, 10, or 15 min before harvesting. 80 μg of extracted protein was used for western blotting with α-PIF3 (1:3000). PIF3-P indicates the phosphorylated PIF3 form. AtTCTP was used as the loading control. Graphs show relative PIF3 levels to AtTCTP in the western blots, assuming the PIF3/AtTCTP level in the dark is 1

When the photoresponses of the *phyB-5* plant were compared with those of the control plant (L*er*) by measuring hypocotyl lengths, *phyB-5* seedlings showed elongated hypocotyls under both continuous R and WL conditions, being 2.1- and 1.9-fold longer than L*er* seedlings, respectively (Fig. 5a). In addition, 4-week-grown *phyB-5* plants flowered earlier than L*er* (Fig. 5b). These phenotypes can be explained by the constitutive shade avoidance responses in the phyB-deficient Arabidopsis.

In general, the elongated growth of plants is regulated with a diurnal cycle, i.e., elongated growth at night and repression in the daylight. Based on PIF3 function for the induction of the elongated growth (Soy et al. 2016), it can be hypothesized that the PIF3 level in the *phyB-5* plant might be higher than that in the control plant (L*er*), especially with the expectation of a greater accumulation of PIF3 at night. To test this hypothesis, seedlings grown in long day conditions were transferred to darkness, and PIF3 accumulation were investigated. The results showed that the protein levels of PIF3 in L*er* increased with the length of incubated time in the dark, demonstrating PIF3 accumulation in the dark or at night (Fig. 5c). Up to 9 h after transfer into darkness, PIF3 accumulation was low. In contrast, PIF3 accumulation in the *phyB-5* plant was much higher than that in the L*er* plant, in which the PIF3 level at 3 h after the transfer into darkness was similar to that at 12–15 h after the transfer in L*er* (Fig. 5d). Accordingly, *YUCCA8* (*YUC8*) and *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*), two genes involved in elongated growth (Kim et al. 2017), were expressed higher in the *phyB-5* plant than in the L*er* plant (Fig. 5e, f). These results were consistent with the observed phenotypes of the *phyB-5* plant. Therefore, the present results help explain why the phyB-deficient plant exhibits elongated growth and suggest the importance of the α-PIF3 antibody in studying the phytochrome function in plants.

Applications for Immunoprecipitation Assays using α-PIF3

In general, antibodies can be used to analyze proteins' behaviors, such as post-translational modifications (e.g.,

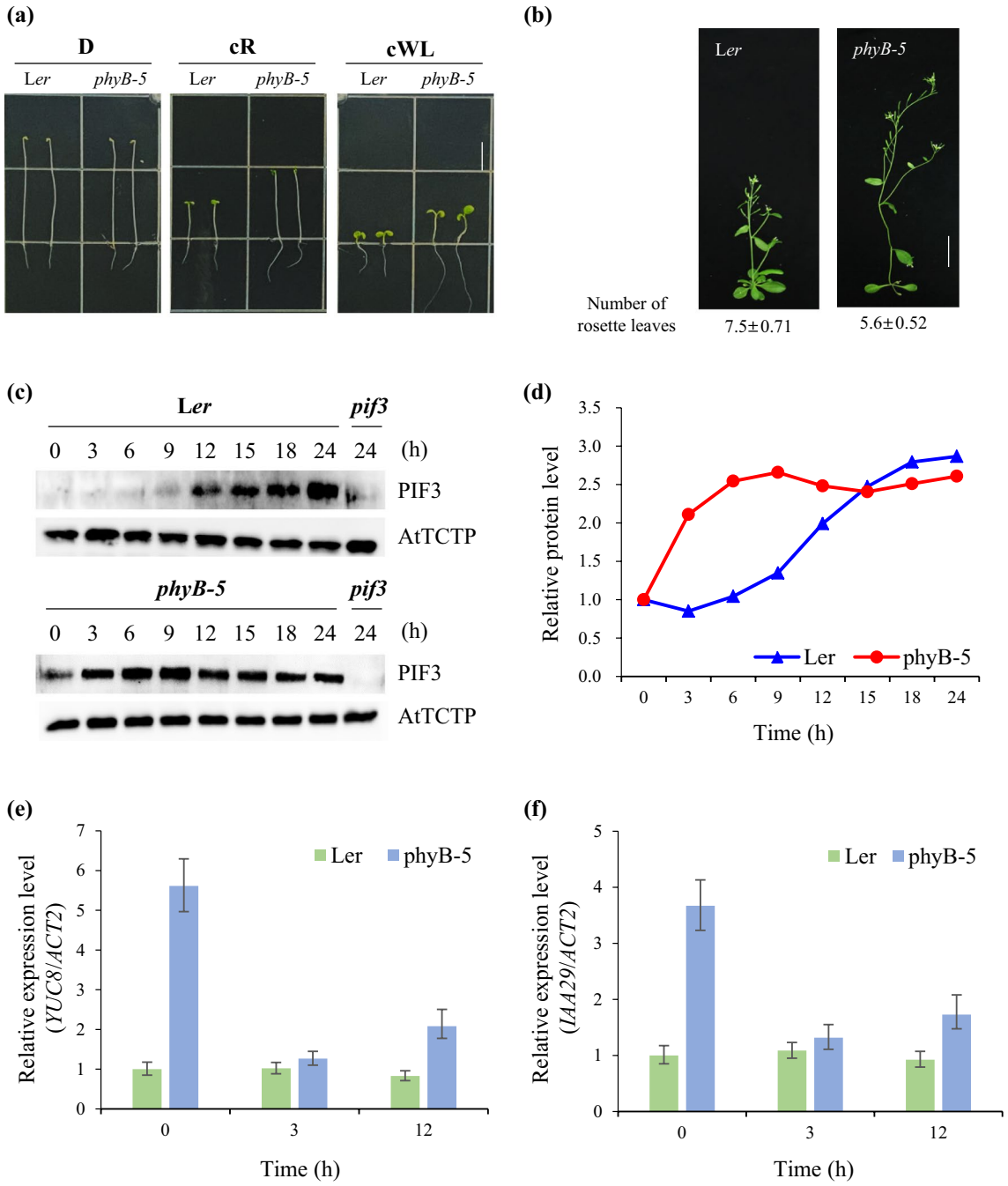

Fig. 5 Analysis of PIF3 accumulation in the dark. **a** Hypocotyl deetiolation of representative L*er* and *phyB-5* seedlings under different light conditions. Seedlings were grown for 4.5 days in the dark (D) or under continuous red (cR, 5 μmol m^{-2} s^{-1}), or continuous white light (cWL, 100 μmol m^{-2} s^{-1}). Scale bar = 5.0 mm. **b** Representative photographs of 4-week-grown plants. Plants were grown in a culture room (21 °C) under long day conditions (16 h light/8 h dark). The numbers of rosette leaves at bolting are shown at the bottom of the images. Scale bar = 3.0 cm. **c** Western blots showing PIF3 accumulation in the dark. 3.5-day-old seedlings grown under the long day conditions (0 h) were transferred into darkness and incubated further for the indicated times. **d** Relative PIF3 levels to AtTCTP in **c**. **e–f** Expression analysis of genes involved in elongated growth. RNA was extracted from 3.5-day-old seedlings grown under long day conditions (0 h) or transferred and incubated in the dark for 3 and 12 h. The transcript levels of *YUC8* (**e**) and *IAA29* (**f**) were analyzed by real-time PCR, using that of *ACT2* for normalization. Relative expression levels were estimated with the transcript level in L*er* at 0 h set to 1, and data are expressed as means ± s.d. ($n = 3$)

phosphorylation), protein–protein interactions (e.g., IP) and protein–DNA interaction (e.g., chromatin IP). Thus, the α-PIF3 antibody was applied for the protein–protein interaction analysis between phyB and PIF3. First, using *phyAB* and phyB:eGFP (transgenic *phyAB* expressing eGFP-fused wild-type phyB) seedlings, a Co-IP was conducted with α-PIF3. In this experiment, total extracts were obtained from seedlings in the dark (for the Pr form of phyB) or under R light (for the Pfr form). Results showed a Pfr-specific interaction of phyB with PIF3 in plants (Fig. 6a). In addition, the PIF3 bands after Co-IP were upper-shifted and smeared, which can be explained by the occurrence of PIF3 phosphorylated forms under R light. Moreover, protein–protein interaction analysis with recombinant proteins was also performed via IP. When the Pr and Pfr forms of recombinant phyB protein were mixed with recombinant PIF3 protein and immunoprecipitated using α-PIF3, the Pfr-specific interaction was obtained (Fig. 6b). Therefore, the α-PIF3 antibody produced in this study can be used as a valuable tool to study the multi-faceted functions of PIF3 in plants.

Conclusions

Among the eight PIF members identified in *A. thaliana*, PIF3 is the first to be discovered to function as a negative regulator in plant photomorphogenesis. After this discovery, the roles of PIF3 in plant growth and development were extensively studied, and expanding roles of PIF3 in multiple processes, such as hormone signaling and cold tolerance, have recently been reported (Jiang et al. 2020; Leivar et al. 2020; Xu and Deng 2020). Thus, the antibody against PIF3 with high sensitivity and specificity is desired to study the multiple functions of PIF3 in plants. In this study, a specific and sensitive polyclonal antibody against PIF3 was successfully produced using recombinant proteins expressed as inclusion bodies. The purified antibody detected only PIF3 but no other PIFs, and exhibited a high sensitivity to detect the endogenous PIF3 level in wild-type Arabidopsis, such as Col-0 and L*er*. Using this antibody, phytochrome-mediated PIF3 phosphorylation and degradation was confirmed, in which PIF3 degradation under FR light was mediated by phyA and that under R light was mediated by other phytochromes, including phyB. Moreover, it was demonstrated that the elongated growth phenotype of phyB-deficient Arabidopsis is positively co-related with the elevated PIF3 level in the plant, especially at night. The antibody was also successfully used to determine the light-dependent interaction of phyB with PIF3 both in vivo and in vitro. Therefore, the PIF3 antibody produced in this study can be used as a powerful tool for elucidating the molecular mechanisms of PIF3 in multiple processes in plants, including photomorphogenesis, hormone signaling, and abiotic/biotic stress responses, such as cold tolerance.

Materials and Methods

Expression of PIF3 as Inclusion Bodies for Antibody Production

Previously, the Arabidopsis α-GI antibody was successfully produced using inclusion bodies (Khaleda et al. 2017). Thus, Arabidopsis PIF3 (At1g09530) was also expressed as inclusion bodies. For this, the *PIF3* gene was subcloned into *pET28a* (Invitrogen) to express the his-tagged recombinant PIF3 protein. The primers and restriction enzymes used for the cloning are listed in Supplementary Table S2. The

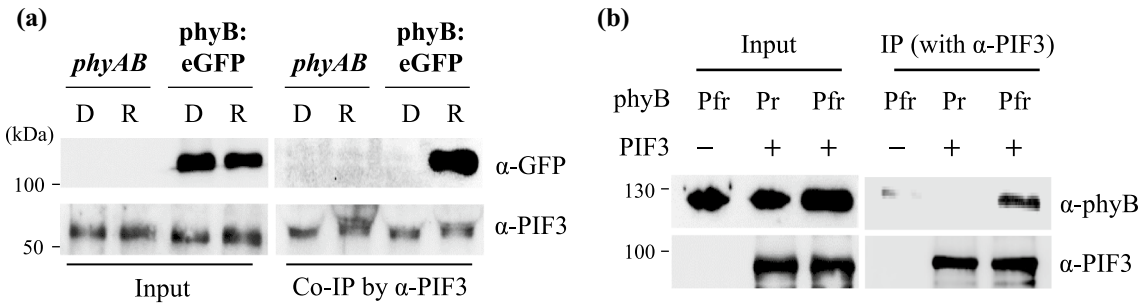

Fig. 6 Interaction analysis between PIF3 and phyB using α-PIF3. **a** Co-immunoprecipitation (Co-IP) analysis. 3.5-day-old dark-grown seedlings of *phyAB* and phyB:eGFP (transgenic *phyAB* with eGFP-fused wild-type phyB) were pretreated with 50 μM MG132 for 4 h, then kept in the dark (D) or exposed to 5 min of red light (R, 10 μmol m^{-2} s^{-1}) before harvesting. Extracted proteins were mixed with the PIF3 antibody-bound Dynabeads™ Protein G beads, and western blotting was then performed with α-GFP and α-PIF3 to detect phyB and PIF3, respectively. **b** In vitro interaction analysis by immunoprecipitation (IP). Pr, red light-absorbing form of phyB (i.e., inactive form); Pfr, far-red light-absorbing form (i.e., active form). 2.0 μg of PIF3 and full-length phyB (either Pr or Pfr) recombinant proteins were mixed with α-PIF3 bound Dynabeads™ Protein G beads, and western blotting was then performed with α-phyB and α-PIF3 to detect phyB and PIF3, respectively

constructs were transformed into *E. coli* strain BL21(DE3)-CodonPlus and expressed as previously described (Shin et al. 2016). After sonicating the cells, the pellet (ppt) was harvested and washed with TE buffer (100 mM Tris, pH 7.8, 1 mM EDTA). Then, the suspended ppt was run on 10% SDS-PAGE and the concentration of the PIF3 inclusion bodies was estimated using BSA as a standard. Overall, 10 mg ppt was used for the elution of PIF3 proteins to produce PIF3 polyclonal antibody (i.e., α-PIF3).

Generation of Polyclonal Antibodies in Rabbits

The PIF3 antibody was produced according to the methods of a previous study (Khaleda et al. 2017). Briefly, PIF3 protein bands were excised from 10% SDS-PAGE gels and eluted using an Electro-Eluter (Bio-Rad). Then, the eluted protein (0.5 mg) was mixed with complete Freund's adjuvant at a 1:1 (v/v) ratio and the antigen was injected into rabbits with triple immunization. Rabbits were housed in accordance with the ethical principles and experimental procedures to minimize suffering, following a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Gyeongsang National University (GNU-151006-B0058). After obtaining anti-serum from the blood samples of the immunized rabbits, the polyclonal antibody was further purified using antigen-specific affinity purification with the recombinant PIF3 protein. For this, the purified PIF3 proteins were separated on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes. After staining with Ponseaus S solution for 1 min, the PIF3 protein bands were excised and blocked with 1% (w/v) BSA in 1 × TBS (50 mM Tris, 150 mM NaCl, pH 7.6). Subsequently, the excised blot strips were mixed with the antiserum (diluted 1 mL antiserum in 9 mL 1% BSA) and incubated overnight at 4 °C. After washing with 1 × TBS, PIF3-bound polyclonal antibodies were eluted with 900 µL of 0.1 M glycine (pH 2.5) and immediately neutralized with 100 µL of 2 M Tris–HCl (pH 8.0). The concentration of the purified PIF3 antibody was approximately 10 µg/µL, and α-PIF3 was stored at 4 °C until further use.

Preparation of *A. thaliana* PIFs

Eight PIFs in *A. thaliana* were expressed and purified to investigate the specificity of the PIF3 antibody produced in this study. For the cloning of *PIF1*, *PIF3*, *PIF4*, *PIF6*, *PIF7*, and *PIF8*, *pGEX 4T-1* (GE Healthcare) with the streptavidin affinity-tag (SAWRHPQFGG; strep-tag) at the 3′ end was used (hereafter, named pStrep). The primers and restriction enzymes used for the cloning are listed in Supplementary Table S2. The *E. coli* strain BL21(DE3)-CodonPlus cells with these constructs were used for the expression of recombinant proteins which were purified by streptavidin affinity chromatography, as previously described (Shin et al. 2016). In this system, the recombinant proteins were expressed with fusions of glutathione *S*-transferase (GST) and strep affinity-tags to their N- and C-termini, respectively (Supplementary Fig. S2). For the cloning of *PIF2*, *PIF3*, and *PIF5*, *pCold TF* (TaKaRa) with 2B8 (RDPLPFFPP; BioJane, Korea) and strep affinity-tags at the 3′ end was used. In this system, the recombinant proteins were expressed with fusions of his-affinity (6 × His) and TF (trigger factor) tags to N-terminus and 2B8- and strep-affinity tags to C-terminus (Supplementary Fig. S2). For the expression of recombinant proteins, *E. coli* strain BL21(DE3)-CodonPlus cells were incubated at 37 °C until OD_{600} reached 0.4–0.6, and then transferred to 15 °C. After incubation for 1 h, IPTG was added to a final concentration of 1 mM and the culture was further incubated overnight at 15 °C. After the cells were harvested and resuspended in ice-cold TE buffer, protein extracts were obtained by repeated sonication of the cells, followed by centrifugation. Then, the supernatant was filtrated with a 0.45 µm microfilter to remove insoluble particles, and the recombinant proteins were purified by streptavidin affinity chromatography. The concentrations of the recombinant proteins were determined using a Quant-iT Protein Assay Kit (Invitrogen).

Western Blot Analysis Using Purified PIF3 Antibody

For western blots with recombinant proteins, 1 µg of each purified protein was run on 10% SDS-PAGE and electroblotted on to a PVDF membrane. Immunoblot analysis was performed with the rabbit α-PIF3 antibody with 1:3000 dilution and peroxidase-conjugated donkey anti-rabbit IgG (Na934v, GE healthcare). The PIF3 protein was detected by chemiluminescence using a Clarity Max Western ECL Substrate (Bio-Rad). For western blots with plant extracts, 10 µL of seeds were germinated and grown for 3.5 days in the dark before collecting the seedlings. The seedlings were then frozen in liquid nitrogen and ground using TissueRuptor (Qiagen) with 250 µL of an extraction buffer (100 mM Tris–HCl, pH 8.0, 100 mM NaH_2PO_4, 8 M Urea, 10 µM MG132, 1 mM PMSF, 1 × protease inhibitor cocktail). The supernatants were used to determine protein concentrations of total plant extracts using a BCA Protein Assay Kit (TaKaRa). Then, 80 µg of the extracts was used for western blot analysis with α-PIF3 (1:3000) to detect PIF3. For loading controls, *A. thaliana* translationally controlled tumor protein (AtTCTP; At3g16640) was immunodetected with the rabbit α-AtTCTP antibody (1:10,000), as previously described (Kim et al. 2012).

Analysis of PIF3 Degradation and Accumulation in Plants

To detect PIF3 in plants, seeds were surface-sterilized and stratified at 4 °C for 3 d in the dark, and were sown on 0.6% phytoagar plates containing half-strength MS salts and vitamins. The seeds were then exposed to white light (WL; 100 μmol m^{-2} s^{-1}) for 4 h to promote germination, returned to darkness (21 °C) for 24 h, and grown for 3.5 days in the dark. The seedlings were then kept in the dark or exposed to R (10 μmol m^{-2} s^{-1}), FR (5 μmol m^{-2} s^{-1}) or WL (100 μmol m^{-2} s^{-1}) for the indicated time. After harvesting seedlings, western blotting was performed with α-PIF3 (1:3000) and α-AtTCTP (1:10,000), as described above. For PIF3 accumulation analysis in plants, after growth under long day conditions (16 h light/8 h dark photoperiod) for 3.5 days, the seedlings under fluorescent light conditions were transferred to dark conditions (set to 0 h) and incubated for sampling. Seedlings were collected at the indicated times and immediately frozen in liquid nitrogen. Then, the plant extracts were used for western blotting to detect PIF3.

Plant Materials

In this study, *pif3* (Col-0 background) and PIF3:eGFP (transgenic Col-0 expressing eGFP-fused PIF3) plants as well as Col-0 were used to analyze the sensitivity of α-PIF3. Among the eight PIFs, the *pif1*, *pif3*, *pif4*, and *pif5* plants were used to analyze the specificity of α-PIF3. Phytochrome-deficient or overexpressing plants, *phyA-201*, *phyB-5*, *phyAB* (*phyA-201phyB-5*), *phyABCDE* (Strasser et al. 2010), A-OX (transgenic *phyA-201* expressing *Avena sativa* phyA; Jeong et al. 2016), and B-OX (transgenic *phyB-5* expressing *A. thaliana* phyB; Jeong et al. 2016), were used to analyze phytochrome-mediated PIF3 degradation. For the comparative analysis of photoresponses between L*er* and *phyB-5*, seedlings were grown at 21 °C for 4.5 days in the dark (D) or under continuous R (cR, 5 μmol m^{-2} s^{-1}) light or continuous WL (cWL, 100 μmol m^{-2} s^{-1}), and images of the seedlings were obtained. To compare growth phenotype, Arabidopsis plants were grown on soil in a culture room (21 °C with a 16-h photoperiod) and flowering time was estimated by counting the leaf numbers at bolting. Images of 4-week-grown plants were then obtained.

Gene Expression Analysis

After growth under long day conditions for 3.5 days, the seedlings under fluorescent light conditions were transferred to darkness and incubated further for 3 and 24 h. Then, RNA was extracted from the seedlings using a FavorPrep™ Plant Total RNA Mini Kit (Favorgen) and cDNA was synthesized from 1 μg of RNA using RNA to cDNA EcoDry™ Premix (TaKaRa). Quantitative real-time RT-PCR analysis was performed using Stratagene MX3005p with Brilliant III Ultra-Fast SYBR Green Q-PCR Master Mix (Agilent Technologies). The transcript levels of *YUC8* and *IAA29* were normalized to that of *ACT2*. The primers used for this analysis are listed in Supplementary Table S2.

Co-Immunoprecipitation (Co-IP)

For Co-IP analysis, 3.5-day-old dark-grown seedlings of *phyAB* and phyB:eGFP (transgenic *phyAB* with eGFP-fused wild-type phyB) plants were pretreated with 50 μM of MG132 for 4 h and then kept in the dark or exposed to R light (10 μmol m^{-2} s^{-1}) for 5 min. Seedlings were immediately frozen in liquid nitrogen and total proteins were extracted using TissueRuptor (Qiagen) in a Co-IP buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Twin 20, 1 mM DTT, 10 μM MG132, 1 mM PMSF, 1 × protease inhibitor cocktail, 10 mM NaF, and 2 mM Na_3VO_4). After centrifugation, supernatants were used for Co-IP. For this, 1 μg of α-PIF3 was incubated with Dynabeads™ Protein G (Invitrogen) for 1 h at 4 °C, and α-PIF3-bound beads were washed with the Co-IP buffer (without 10 μM MG132, 1 mM PMSF, 1 × protease inhibitor cocktail, 10 mM NaF, and 2 mM Na_3VO_4) three times. Subsequently, the supernatants were mixed with the washed α-PIF3-bound beads for 30 min at 4 °C. After washing with a buffer (1 × PBS, 1% Triton X-100, 3% BSA) three times, SDS loading buffer was added and western blot analysis was performed with α-PIF3 (1:3,000) and α-GFP (1:5,000; sc-9996, Santa Cruz Biotechnology).

In vitro Protein–Protein Interaction Analysis

For the interaction analysis between phyB and PIF3 in vitro, recombinant protein of full-length strep-tagged phyB was expressed in *Pichia pastoris* cells and purified by streptavidin affinity chromatography, as previously described (Shin et al. 2014, 2016). Then, Pr and Pfr were prepared by irradiating FR (50 μmol m^{-2} s^{-1} for 15 min) or R light (20 μmol m^{-2} s^{-1} for 30 min). For in vitro interaction analysis by IP, phyB (2.0 μg) and PIF3 (2.0 μg) were mixed and incubated for 30 min at 4 °C in 500 μL of 1 × TBS, and α-PIF3-bound Dynabeads™ Protein G beads were added and incubated for an additional 30 min. PIF3 and phyB in the input and immunoprecipitated fractions were detected using Clarity Max Western ECL Substrate (Bio-Rad) with α-PIF3 (1:3000) and α-phyB (1:2000; aN-20, Santa Cruz Biotechnology).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12374-021-09302-9.

Acknowledgements This research was supported under the framework of international cooperation program (NRF Grant no. 2019K2A9A1A06100097 to J.-I.K), and by the Basic Science Research program (NRF Grant no. 2018R1A6A3A11045293 to Y.-J.H.) managed by the National Research Foundation of Korea, and in part by the Next-Generation BioGreen21 Program from Rural Development Administration, Republic of Korea (TAGC Grant no. PJ01325301). We would like to thank Editage (www.editage.co.kr) for English language editing.

Author Contributions YJH and JIK designed the project, WYK produced the polyclonal antibody, and DMC and JYC performed the experiments. YJH, DMC, JYC and JIK analyzed the data and discussed about the results. YJH and JIK wrote the paper, and all authors approved the manuscript.

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