BR-dependent phosphorylation modulates PIF4 transcriptional activity and shapes diurnal hypocotyl growth

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Signaling by the hormones brassinosteroid (BR) and gibberellin (GA) is critical to normal plant growth and development and is required for hypocotyl elongation in response to dark and elevated temperatures. Active BR signaling is essential for GA promotion of hypocotyl growth and suppresses the dwarf phenotype of GA mutants. Cross-talk between these hormones occurs downstream from the DELLAs, as GA-induced destabilization of these GA signaling repressors is not affected by BRs. Here we show that the light-regulated PIF4 (phytochrome-interacting factor 4) factor is a phosphorylation target of the BR signaling kinase BRASSINOSTEROID-INSENSITIVE 2 (BIN2), which marks this transcriptional regulator for proteasome degradation. Expression of a mutated PIF41A protein lacking a conserved BIN2 phosphorylation consensus causes a severe elongated phenotype and strongly up-regulated expression of the gene targets. However, PIF41A is not able to suppress the dwarf phenotype of the bin2-1 mutant with constitutive activation of this kinase. PIFs were shown to be required for the constitutive BR response of bes1-D and bzz1-1D mutants, these factors acting in an interdependent manner to promote cell elongation. Here, we show that bes1-D seedlings are still repressed by the inhibitor BRZ in the light and that expression of the nonphosphorylatable PIF41A protein makes this mutant fully insensitive to brassinazole (BRZ). PIF41A is preferentially stabilized at dawn, coinciding with the diurnal time of maximal growth. These results uncover a main role of BRs in antagonizing light signaling by inhibiting BIN2-mediated destabilization of the PIF4 factor. This regulation plays a prevalent role in timing hypocotyl elongation to late night, before light activation of phytochrome B (PHYB) and accumulation of DELLAs restricts PIF4 transcriptional activity.

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De-etiolation involves a drastic change in plant morphology as germinating seedlings shift from dark to the light. In darkness, hypocotyl growth is rapidly induced at the expense of the cotyledons that remain folded, whereas the upper part of the hypocotyl forms an apical hook that protects the shoot apical meristem during rapid elongation in soil. Light arrests hypocotyl elongation and promotes opening and expansion of the cotyledons at the time that induces the differentiation of chloroplasts to start photosynthetic life. This photomorphogenic response is mediated through the activation of different families of photoreceptors, of which the red/far-red-absorbing phytochromes (PHYs) are the best characterized (Quail 2010). PHYs regulate light responses by inhibiting the PIFs (PHY-interacting factors), a family of basic helix-loop-helix (bHLH) transcriptional regulators that promote etiolated growth in the dark (Leivar and Quail 2011). Although initially regarded as negative regulators of PHYB signaling, these factors are now recognized to function as main regulators of cell elongation and shade avoidance responses (Castillon et al. 2007; Nozue et al. 2007; De Lucas et al. 2008; Feng et al. 2008; Oh et al. 2009) by binding to G-box elements (CACGTG) and directly activate the expression of a large number of genes (Hornitschek et al. 2012; Zhang et al. 2013). Interaction with the Pfr form of PHYs induces phosphorylation of PIFs and marks these factors for proteolytic degradation, thus reversing PIF
transcriptional activity and promoting light-regulated gene expression [Shen et al. 2007; Lorrain et al. 2008]. Consistent with this model of action, pif1 pif3 pif4 pif5 quadruple mutants [pifq] display a constitutive photomorphogenic response in the dark and ectopic expression of chloroplast-related genes [Leivar et al. 2008].

Isolation of mutants with a de-etiolated response in the dark has also evidenced a role of the plant hormones gibberellin [GA] and brassinosteroid [BR] in suppressing light signaling. Mutants impaired in biosynthesis or signaling of these hormones have a characteristic dwarf phenotype and, in darkness, display short hypocotyls, an open apical hook, and activated expression of light-regulated genes as if they had received light [Alabadi et al. 2004; Nemhauser and Chory 2004]. Loss-of-function mutations in the DELLA RGA and GAI genes suppress the de-etiolated phenotype of ga1-3 seedlings, thus demonstrating that constitutive photomorphogenesis in these GA-deficient mutants is caused by the accumulation of DELLAs [Alabadi et al. 2004]. This family of nuclear repressors plays a central role in inhibiting GA-regulated gene expression [Fleet and Sun 2004] and is destabilized in the dark [Achard et al. 2007]. GA sensing by the cytosolic receptor GID1 changes the receptor conformation and promotes the interaction of the GA–GID1 complex with the DELLAs [Murase et al. 2008; Shimada et al. 2008]. Formation of a GA–GID1–DELLA complex favors ubiquitination of these repressors by the E3 ligase SCFSLY [Dill et al. 2004; Fu et al. 2004] and marks them for proteasomal degradation, allowing GA-induced hypocotyl growth. Work by our group and other groups has established that DELLAs bind the bHLH DNA recognition domain of PIFs, sequestering these factors into an inactive complex unable to bind to DNA [De Lucas et al. 2008; Feng et al. 2008; Gallego-Bartolome et al. 2010]. Nuclear balance of PIFs and DELLAs is therefore crucial to sustain the etiolated state of dark-grown seedlings and light-mediated degradation of PIFs together with the stabilization of DELLAs, providing a rapid mechanism to repress transcriptional activity of these factors during photomorphogenesis.

Etiolated growth also involves a prominent role of BR signaling, since BR synthesis and response mutants display a stronger de-etiolated phenotype in the dark than GA mutants [Clouse 2011]. BR application rescues the germination phenotype of GA-insensitive mutants [Steber and McCourt 2001] and has a synergistic effect with GAs on hypocotyl elongation [Tanaka et al. 2003], suggestive of a cross-talk interaction of these hormones. In Arabidopsis, the BR signal transduction cascade is by now well understood, thanks to extensive molecular and genetic studies that contributed to the identification of all major signaling components in the pathway [Wang et al. 2012]. BR’s perception by the membrane receptor kinase BRASSINOSTEROID-INSENSITIVE1 (BRI1) [Kinoshita et al. 2005; She et al. 2011; Santiago et al. 2013] promotes association of this receptor kinase with the coreceptor BRI1-ASSOCIATED KINASE 1 (BAK1) [Nam and Li 2002; Wang et al. 2008] and induces a phosphorylation cascade that leads to activation of BRI1 SUPPRESSOR 1 (BSU1) [Mora-Garcia et al. 2004; Kim et al. 2009]. This PP1-type phosphatase dephosphorylates the GSK3-like kinase BRASSINOSTEROID-INSENSITIVE 2 (BIN2) and inactivates this enzyme [Li and Nam 2002; Kim and Wang 2010]. BIN2 has a negative regulatory function in BR signaling by phosphorylating two homologous transcription factors, BRI1-EMS SUPPRESSOR 1 (BES1) and BRASSINAZOLE-RESISTANT 1 (BZR1), to inhibit their transcriptional activity [He et al. 2002; Yin et al. 2002]. When BR levels are elevated, BIN2 is inactivated, and BES1 and BZR1 are dephosphorylated by PP2A [Tang et al. 2011], the nonphosphorylated forms of these factors being shown to be more stable and to accumulate in the nucleus, where they bind the promoters of multiple genes and regulate their expression in a BR-dependent fashion [He et al. 2005; Yin et al. 2005; Sun et al. 2010].

Although BZR1 was originally identified as a repressor of BR synthesis [He et al. 2002; Wang et al. 2002] and BES1 was reported to heterodimerize with the BIM1 factor to activate gene expression [Yin et al. 2002, 2005], more recent studies showed that these factors bind similar BRRE and E-box elements and act as both activators and repressors, depending on the gene target [Sun et al. 2010; Yu et al. 2011]. BZR1 has been recently shown to bind PIF4 and function as a PIF4 coactivator, with the BZR1–PIF4 complex binding a G-box motif identical to that bound by the PIF4 factor and synergistically activating a common set of genes [Oh et al. 2012]. BES1 and BZR1 also interact with the DELLAs, these repressors blocking BES1 and BZR1 DNA-binding activity in a manner similar to that reported for PIF4 [Bai et al. 2012; Gallego-Bartolomé et al. 2012; Li et al. 2012]. Interestingly, BZR1-dependent GA-regulated genes largely overlap with the BZR1–PIF4 coactivated genes [Bai et al. 2012; Oh et al. 2012], suggesting that DELLAs’ interaction with the BES1/BZR1 phosphorylation domain may compete for BZR1–PIF4 complex formation. Here, we show that the GSK3-like kinase BIN2, besides inactivating the BES1/BZR1 factors, phosphorylates PIF4/PF5 to mark these transcription factors for proteasome degradation. This regulation is especially relevant late in the night, when PIF4 transcription is up-regulated and the protein is stable. Although BES1 and BZR1 were reported not to be significantly affected by light [Luo et al. 2010], the active nonphosphorylated forms of the PIF4 and BES1/BZR1 factors overlap only during late night, BES1/BZR1 coactivator function thus having a relevant role in timing PIF4 transcriptional activity and in rhythmic hypocotyl growth. These results confirm a main function of the PIFs and BES1/BZR1 factors in cell elongation and highlight a role of these two families of bHLH factors as main integrators of light, BR, and GA signals by means of PHYB- and BIN2-induced destabilization and inactive complex formation with the DELLAs.

Results

Active BR signaling is required for GA promotion of hypocotyl growth

GA-insensitive sly1-10 and gai1 mutants exhibit seed germination defects, a phenotype that is alleviated by treatment with brassinolide [BL] [Steber and McCourt稔
De Lucas et al. 2008; Nemhauser et al. 2004, 2006; Vert et al. 2005) and in dark-grown seedlings (Sun et al. 2010).

In line with such a BR-related phenotype, a significant overlap is observed between the genes differentially expressed in *Pif4*-OE lines grown in red light (De Lucas et al. 2008) and in dark-grown *pifq* seedlings (for dark data set, see Leivar et al. 2009; for *pif4* data set, see Zhang et al. 2013) and those induced by BL treatment (Goda et al. 2004, 2008; Nemhauser et al. 2004, 2006; Vert et al. 2005) or down-regulated in *br1-116* seedlings (Sun et al. 2010). As shown in Figure 1E, 24.9% of the up-regulated genes in *Pif4*-OE seedlings and 21.1% of the genes down-regulated in dark-grown *pifq* seedlings are positively regulated by BL (4.8% expected randomly). Notably, genes involved in cell wall modification (*pectinesterases, XTHs, and expansins*) and auxin response (*SAURs* and *Aux/IAA*) are highly enriched among the coregulated genes, in agreement with recent studies establishing that PIF4 and BZR1 cooperatively regulate these gene targets (Oh et al. 2012).

**PIF4 interacts with the BR signaling kinase BIN2**

The GSK3-like kinase BIN2 negatively regulates BR signaling by inhibiting BES1/BZR1 transcriptional activity. BIN2-mediated phosphorylation of these factors inhibits DNA binding and promotes BES1/BZR1 interaction with cytosolic 14-3-3 proteins in addition to induce their degradation by the proteasome (Zhao et al. 2002; Vert and Chory 2006; Gampala et al. 2007). BR signaling
Figure 1. BR signaling overcomes the growth-repressing effects of DELLAs. (A) Hypocotyl lengths of 5-d-old wild-type (Col-0) and det2-1 seedlings grown on MS medium supplemented with mock solution (mock), 0.5 μM BRZ, 0.5 μM BRZ + 25 μM GA3 [BRZ+GA], 25 μM GA3 [GA], 1 μM cpi-BL + 25 μM GA3 [BL+GA], and 1 μM cpi-BL + 0.5 μM PAC [BL+PAC]. BRZ-treated wild-type seedlings and the BR-deficient det2-1 mutants are insensitive to GA application. Combined application of GA+BL leads to exaggerated hypocotyl growth of wild-type and det2-1 seedlings. (B) GA-induced destabilization of the GFP-RGA fusion in 5-d-old det2-1 pRGA∷GFP-RGA seedlings. Application of the inhibitor PAC leads to increased levels of accumulation of this DELLA and additional growth inhibition of these plants. GA induces degradation of the RGA repressor but does not promote growth of the BR-deficient seedlings. (C) BL-induced stabilization of the PIF4-HA protein. An anti-HA antibody was used for Western immunoblot detection of the PIF4-HA protein. (Top panel) Five-day-old dark-grown 35S∷PIF4-HA seedlings were kept on MS medium (mock) or treated with 1 μM BL (BL), 0.5 μM BRZ (BRZ), or 0.5 μM BRZ + 0.5 μM MG132 [BRZ+MG132] for the indicated times. A faster-migrating band of the protein is found to be enriched in the BL-treated plant. (Bottom panel) Five-day-old dark-treated seedlings were kept on MS medium (mock) or treated with 1 μM BL (BL), 0.5 μM BRZ (BRZ), or 0.5 μM BRZ + 10 μM MG132 [BRZ+MG132] for the indicated times. A faster-migrating band of the protein is found to be enriched in the BL-treated plant. (Top panel) Five-day-old dark-grown seedlings were kept on MS medium (mock) or treated with 1 μM BL (BL), 0.5 μM BRZ (BRZ), and total protein extracts were incubated for 1 h with (+) or without (−) calf intestinal phosphatase (CIP). A fraction of the extract was sampled before incubation (T0). Slower-migrating forms of the protein disappear after CIP treatment, indicating that they correspond to phosphorylated forms of PIF4. (D) BL response phenotype of PIF4OX and phyB plants. Seedlings were grown in the light on vertical MS plates supplemented with mock solution (mock), 0.5 μM BRZ [BRZ], 0.5 μM BRZ + 25 μM GA3 [BRZ+GA], or 25 μM GA3 [GA], and hypocotyl lengths were measured (n = 20) at day 5 after germination. Error bars are ±SEM. (E) PIF4-induced genes are positively regulated by BRs. Venn diagram showing the overlap among genes induced in PIF4OX transgenic plants or repressed in the pifq mutant and BR-regulated genes (induced by BL treatment or repressed in the bri1-116 mutant). Clustering analysis of FC expression of the genes coregulated in PIF4OX plants grown in red light [PIF4OX] with 3 h of BL treatment [BL] and the bri1-116 mutant [bri1-116] and pifq seedlings grown in the dark [pifq] (yellow) up, [purple] down.
inactivates BIN2 kinase activity via BSU1-mediated dephosphorylation at Tyr200, enabling nuclear accumulation of the nonphosphorylated BES1/BZR1 factors and BR-activated gene expression [Kim and Wang 2010]. Thus, BIN2 is a good candidate to regulate PIF4 levels, as inactivation of this kinase by BL may result in the fastest-migrating form of this factor seen in BL-treated seedlings (Fig. 1C). A direct interaction of the full-length BIN2 protein and PIF4 was actually observed in yeast two-hybrid assays [Fig. 2A]. Deleted versions of the PIF4 protein mapped the BIN2 interaction domain to a fragment immediately C-terminal to the bHLH dimerization domain, as a PIF4 truncated version from Val312 to the C-terminal end still interacts with BIN2 [Fig. 3D]. Deletion of this C-terminal end, however, does not abrogate binding, suggesting that an additional interacting domain located between residues 107 and 312 cooperates with the C-terminal region to enhance BIN2-binding affinity [Fig. 3D].

Direct interaction of these proteins was confirmed by in vitro pull-down assays in which the 35S-Met-labeled PIF4 and BES1 were found to be retained with similar affinities by agarose beads with the immobilized GST-BIN2 protein [Fig. 2B]. Bimolecular fluorescence complementation [BiFC] and coimmunoprecipitation [co-IP] studies in Nicotiana benthamiana leaves further confirmed this interaction in vivo. Nuclear fluorescence of reconstituted split YFP was observed in leaves cotransfected with the BIN2-eYFC and PIF4-eYFN fusions but not in control leaves expressing the eYFC-BIN2 and eYFN or the eYFC and PIF4-eYFN proteins [Fig. 2C]. The PIF4-GFP protein was also pulled down out of plant extracts coexpressing the BIN2-HA and PIF4-GFP proteins after immunoprecipitation of BIN2-HA [Fig. 2D], confirming interaction of these proteins in plant cells.

\[ \text{BIN2 phosphorylates PIF4 in vitro} \]

In vitro kinase assays using the purified GST-BIN2, PIF4-6xHis, and MBP-BES1 proteins showed that BIN2 is able to phosphorylate both BES1 and PIF4 [Fig. 3A]. Labeling of these factors is only observed when BIN2 is added to the reaction mix. Also, although in pull-down assays BIN2 bound with similar affinities the BES1 and PIF4 proteins, labeling of PIF4 was weaker than that of the BES1 protein. This would suggest that PIF4 has fewer phosphorylation sites than BES1, reported to be modified by this kinase in at least 10 independent Ser/Thr residues [Ryu et al. 2010]. Incubation with the specific inhibitor bikinin [De Rybel et al. 2009] blocked PIF4 phosphorylation as well as autophosphorylation of BIN2, confirming that labeling of this protein is mediated by the BIN2 kinase [Fig. 3C]. Therefore, we can conclude that PIF4 is a bona fide substrate for the BR signaling kinase BIN2.

Mutation of a conserved BIN2 phosphorylation consensus leads to strong PIF4 stabilization

GSK3-kinases phosphorylate Ser/Thr residues in the short consensus motif (S/T)-X-X-X-(S/T). Identification of the BES1/BZR1 factors as main BIN2 phosphorylation substrates revealed that this plant kinase phosphorylates Ser/Thr residues within the same motif [Ryu et al. 2010]. However, in contrast to animal GSK3-kinases, it does not require a priming phosphorylation event to act on these proteins [Peng et al. 2010]. In a search for residues that match this consensus motif in the PIF4 protein, we identified >10 putative phosphorylation sites [Supplemental Fig. S5]. We reasoned that if these sites are important to modulate PIF4 stability, they should be conserved in all PIF4 orthologs. Alignment of the Arabidopsis protein with its closest homologs in other species actually
kinase inhibitor bikinin. Addition of 2 μM and 20 μM bikinin to the phosphorylation mix represses PIF4 phosphorylation and the autophosphorylation activity of BIN2. [D] Mapping of the BIN2-interacting domain. Schematic representation of the PIF4 domains and the deletions used in the yeast two-hybrid assay are shown. [E] Deletions of the PIF4 protein were cotransformed into yeast cells with the BIN2-BD construct to map the interacting domain. A C-terminal fragment lacking the bHLH domain binds the BIN2 protein, although with lower affinity. Removal of this C-terminal region does not abrogate binding, indicating that the HLH region contributes to this interaction.

identified two consensus motifs that are conserved in all PIF4/PIF5 proteins across evolutionarily divergent species (Supplemental Fig. S5). Site-directed mutagenesis of the conserved T\textsuperscript{S164}VGPS\textsuperscript{S168} motif to replace the three Ser/Thr residues by Ala, hence generating a non-phosphorylatable PIF4A1 mutant protein (Fig. 3B), led to a strong stabilization of this factor (Fig. 4B,D) and reduced the efficiency of in vitro labeling by BIN2 (Fig. 3A), although it did not fully abolish phosphorylation. Residual labeling by this kinase is likely to be due to phosphorylation of the second conserved motif or secondary modification of the nonconserved sites found in the protein. However, further mutation of Ser\textsuperscript{T160} to Ala to disrupt this second conserved motif did not lead to protein stabilization, indicating that this motif is less relevant for PIF4 function.

Consistent with these results, lines overexpressing the PIF4A1-GFP protein fusion were severely elongated and showed extremely early flowering [Fig. 4A], as anticipated for a strong stabilization of this factor. Confocal GFP fluorescence and Western blot studies confirmed higher levels of accumulation of this mutant protein fusion in the light [Fig. 4B,D], as compared with 35S:PIF4-GFP lines showing equivalent transcript levels for the wild-type protein fusion [Fig. 4C]. In dark-grown seedlings, differences in protein levels were smaller due to additional stabilization of the wild-type protein [Fig. 4D]. However, subcellular localization of the PIF4A1-GFP protein was not altered with respect to PIF4-GFP. Both the PIF4-GFP and PIF4A1-GFP fusions show a diffuse nuclear expression and form similar nuclear speckles upon red light irradiation (Bauer et al. 2004, Leivar et al. 2008), with a pulse of far-red light reversing speckle formation in both proteins (Supplemental Fig. 6). BL treatment, on the other hand, did not alter their subcellular localization [Supplemental Fig. 6].

35S:PIF4A1-GFP lines display up-regulated expression of the PIL1 transcript and respond to BL by enhanced levels of activation of this gene (Fig. 4E). When treated with BRZ, PIL1 gene expression was also found to be less suppressed in these plants than in 35S:PIF4-GFP lines [Fig. 4E], suggestive of a stabilization of the PIF4A1 protein irrespective of BIN2 kinase activation.

PIF4A1 expression partially suppresses the dwarf phenotype of bin2-1 mutants

To further assess constitutive activation of this mutant protein, we analyzed whether PIF4A1 overexpression suppresses the severe dwarf phenotype of bin2-1 mutants, impaired in BR signaling due to constitutive BIN2 activation [Peng et al. 2008]. As shown in Supplemental Figures S9 and S10, PIF4A1 expression rescued the semidwarf phenotype of heterozygous bin2-1\textsuperscript{77} mutants but did not suppress the more severe phenotype of homozygous plants. PIF4A1 bin2-1 seedlings are slightly taller than the bin2-1 mutant [Supplemental Fig. S10] but still display the characteristic curled leaf and severe dwarf phenotype of these mutants. Such dosage complementation effects indicate that, in addition to PIF4, other BIN2-regulated factors are required for hypocotyl growth, in agreement with the recent finding that BZR1 binds G-box elements identical to those bound by PIF4 in the promoters of cell
elongation genes (Oh et al. 2012). PIFs are actually required for BZR1-induced cell elongation, with loss-of-function mutations of the PIF1, PIF3, PIF4, and PIF5 genes in the pifq mutant suppressing the elongated phenotype of the constitutive bzi1-1D mutant (Oh et al. 2012). Hence, concurrent activity of the BZR1 and PIF4 factors is essential for cell elongation. The observation that bzi1-1D suppresses the dwarf phenotype of bri1-116 seedlings in the dark but not in the light and that light-grown PIF4OX bzi1-1D bzi1-116 seedlings are shorter than PIF4OX lines indeed supports this regulatory model and demonstrates that phosphorylation by BIN2 mediates destabilization of this factor in the light. 

**BIN2 phosphorylation plays a main role in destabilizing the PIF4 protein at dawn**

Growth of young seedlings follows a rhythmic elongation pattern such that, in short days, rapid hypocotyl growth occurs at dawn and alternates with an interval of reduced elongation during daytime and early night (Nozue and Maloof 2006). Thus, we wanted to test whether PIF4 phosphorylation plays a role in defining this window of active cell elongation. To this aim, we generated transgenic pif4pif5 lines that expressed the wild-type PIF4-HA and mutant PIF41A-HA proteins under the control of its native promoter. We selected lines in which the levels of expression of these transgenes were similar to Col-0 and verified that transcript accumulation in these plants [Fig. 5D] followed the diurnal expression pattern reported for the endogenous PIF4 gene (Nozue et al. 2007; Yamashino et al. 2013). Lines generated for the wild-type PIF4-HA protein actually had hypocotyl lengths similar to those of Col-0 plants and showed a wild-type response to PAC (Supplemental Fig. S12) and BRZ treatments (Fig. 7A, B, below). When grown at 28°C, these plants also showed an elongation growth similar to that observed for Col-0 seedlings (Supplemental Fig. S7). PIF41A-HA lines, in contrast, had taller hypocotyls and showed the characteristic elongated petioles and narrower leaves of plants grown in the shade [Figs. 5A, 7A, B, below], indicating that phosphorylation by BIN2 mediates destabilization of this factor in the light. 

**Figure 4. PIF41A overexpression causes a severely elongated phenotype and activated PIL1 gene expression in the presence of BRZ.** (A) Overexpression of the PIF41A mutant protein causes severe elongation of the petioles and stem and an extremely early flowering phenotype. Plants were grown for 3 wk. (B) Detection of the PIF1A-GFP protein in roots of 5-d-old plants. Nuclear fluorescence in PIF41A-GFP plants is stronger than in PIF4-GFP seedlings, indicating a higher stability of the PIF41A protein. (C) Quantification of PIF4 transcript levels in the PIF4-GFP and PIF41A-GFP overexpression lines. Plants were grown in continuous light for 5 d. Transcript levels were equivalent in both lines. (D) Western blot detection of the PIF4-GFP and PIF41A-GFP proteins. Plants were grown for 5 d under light or dark conditions. Higher levels of PIF41A are detected in light-grown seedlings, implicating that phosphorylation by BIN2 mediates destabilization of this factor in the light. (E) Quantitative real-time PCR analysis of PIL1 transcript levels in Col-0 and in lines overexpressing the PIF4 and PIF41A proteins. Plants were grown in continuous light for 5 d. An enhanced activation of PIL1 in response to BL was observed in PIF41A seedlings. (*) $P < 0.05$ by Student’s t-test. Error bars are ±SD.
dropped after dusk, when PIF4 transcription is repressed (Fig. 5B,D; Nusinow et al. 2011). A related daytime pattern of protein accumulation was also recently reported for a PIF4-citrine-HA construct (Yamashino et al. 2013), although the taller phenotype of these plants did not exclude an additional stabilization of the protein due to the C-terminal citrine tag. Our HA-tagged lines display a hypocotyl length similar to that of Col-0, and relatively high levels of the protein were detected during daytime. Furthermore, in lines expressing the same PIF4-HA fusion under the control of the 35S promoter (Supplemental Figure S11), a preferential accumulation of the protein was seen at night, as previously reported (Nozue et al. 2007). This indicates that although stability of PIF4 is higher in darkness, active transcription of this gene may lead to substantial levels of accumulation of this factor during daytime, depending on light irradiance. However, during this phase of the day, PIF4 is repressed by the DELLAs, which were reported to accumulate in the light (Achard et al. 2007). It is also noteworthy that increased PIF41A stability during late night correlates with preferential accumulation as a faster-migrating form of the protein (Figs. 5B, 1C), although this mutation does not preclude formation of slower isoforms, suggesting that PIF4 is phosphorylated by other kinases in addition to BIN2. Indeed, upon light exposure, slower-migrating forms are observed for both the wild-type and PIF41A proteins (Fig. 5B), suggesting that PIF4 is subjected to phosphorylation by a PHYB-regulated kinase, as reported for PIF3 (Ni et al. 2013).

To estimate the stabilization of this mutant protein, we treated PIF4 and PIF41A seedlings with cycloheximide (CHX) and with CHX and BL (Fig. 6A). In these studies, PIF4 is destabilized by 30 min of CHX application, while PIF41A is still detectable after 90 min of CHX treatment. Also, upon BL application, PIF4 half-life was similar to that of the PIF41A protein, whereas this hormone did not have any effect on PIF41A protein levels (Fig. 6A). Consistent with these results, when PIF4 and PIF41A lines were crossed to the bin2.1 mutant, we observed that constitutive activation of BIN2 in these seedlings leads to a strong reduction in PIF4 protein levels but has only a minor effect on PIF41A protein stability (Fig. 6B).

We also assessed whether this mutation affects rhythmic plant growth by continuously measuring the growth of Col-0, PIF4-HA, PIF41A-HA, and PIF4OX seedlings during 2 d (Fig. 6C). Under short days, growth of Col-0 and PIF4-HA was maximal at dawn, while PIF4OX seedlings showed an increased growth rate during early night, as previously described (Nozue et al. 2007). Remarkably, PIF41A seedlings elongated also during daytime (Fig. 6C), indicating that BL-dependent phosphorylation of the PIF4 factor plays an important role in modulating rhythmic hypocotyl growth.
PIF41A bes1-D seedlings are insensitive to BRZ in the light

The gain-of-function bes1-D and bzr1-1D mutations cause constitutive BES1 and BZR1 activation due to identical mutations in the PEST domain, which enhance PP2A-binding affinity and lead to increased dephosphorylation of these factors [Tang et al. 2011]. These mutants show a BRZ-insensitive response in the dark but opposite phenotypes in the light, although the BES1 and BZR1 proteins share 88% overall identity. While the bzr1-1D mutant displays a weak dwarf phenotype and a hypersensitive response to BRZ in the light, light-grown bes1-D seedlings are largely insensitive to this inhibitor. This phenotype is at present not well understood, as light does not seem to affect levels of these proteins [Luo et al. 2010]. Since BES1 and BZR1 promote cell elongation by formation of a coactivator complex with PIF4 [Oh et al. 2012], it is possible that the hypersensitive response to BRZ of bzr1-1D seedlings results from an increased phosphorylation and destabilization of the PIF4 factor in the light. However, the BRZ-insensitive response of light-grown bes1-D mutants does not fit well with this model, unless these plants still show a reduction in hypocotyl growth on BRZ. As shown in Figure 7, A and B, bes1-D seedlings are in fact shorter on BRZ than in mock medium and display reduced levels of expression of the PIL1 and PRE5 target genes [Fig. 7C]. Also, they are only slightly taller than Col-0, although BES1 is constitutively activated in this mutant. PIF41A lines, in contrast, show a much elongated phenotype but still respond to BRZ by a reduction in hypocotyl growth, indicating that the non-phosphorylatable PIF41A protein requires BES1/BZR1 function to promote cell elongation. Therefore, we introduced the bes1-D gain-of-function mutation into the pPIF4-PIF41A lines to test whether combined expression of these two mutant proteins confers a BRZ-insensitive response in the light. Light-grown PIF41A bes1-D seedlings actually show elongated hypocotyls on BRZ similar to those in mock treatments [Fig. 7B], demonstrating that the BIN2-insensitive forms of PIF4 and BES1 are required for hypocotyl growth in the light. Gene expression quantitative PCR (qPCR) studies confirmed that expression of both PIF41A and bes1-D proteins leads to constitutive up-regulated levels of expression of the PIL1, XTR7, and PRE5 target genes on BRZ [Fig. 7C]. Taken together, these results demonstrate that BR signaling plays a prevalent role in hypocotyl elongation by suppressing BIN2 kinase activity, with inactivation of this kinase allowing stabilization of the PIF4 factor and BES1/BZR1 nuclear accumulation, with a concerted activation of these two families of regulators being essential for growth.

Discussion

PIFs play a pivotal role in cell elongation by directly activating the expression of genes with a role in cell wall loosening and auxin-related signaling [Leivar and Quail 2011]. Members of this gene family play a redundant role in seedling etiolation and loss of function of the PIF1, PIF3, PIF4, and PIF5 genes in the pifq mutant, leading to a dwarf de-etiolated phenotype in the dark [Leivar et al. 2009]. PIF4 was recently shown to interact with BES1/BZR1 and act in an interdependent way with these factors to activate expression of multiple BR-regulated genes [Oh et al. 2012]. In this study, we disclose a role of BIN2 in destabilization of the PIF4 factor and show that this modification plays a key role in timing seedling elongation to the end of the night. We show that active BR signaling is required for growth promotion in response to DELLA destabilization and that BR application promotes accumulation of a faster-migrating form of the PIF4 protein; suggestive of a role of BRs in modulating phosphorylation of this factor. PIF4 binds the negative BR signaling kinase BIN2 in yeast cells, and interaction of these proteins was confirmed in vivo by BiFC and co-IP assays. BIN2 phosphorylates the PIF4 protein in vitro, and we show that this modification plays an important role in modulating stability of this factor.

BR signaling stabilizes the PIF4 factor

Expression of the PIF41A mutant protein lacking a conserved BIN2 phosphorylation consensus leads to an elongated phenotype and reduced sensitivity to the BR biosynthetic inhibitor BRZ. However, this mutation suppresses only in part the dwarf phenotype of the BR-insensitive bin2-1 mutant, indicating that other BIN2-regulated factors also play an essential role in hypocotyl growth. BIN2 phosphorylates the HLH BES1/BZR1 factors at multiple sites, the modified BES1/BZR1 proteins being unable to bind to DNA and be retained in the cytosol via interaction with the 14-3-3 phosphopeptide-binding proteins, where they are degraded by the proteasome [Zhao et al. 2002; Vert and Chory 2006; Gampala et al. 2007]. BZR1 was shown to interact with PIF4 and bind as a complex with this factor to the promoters of several genes [Oh et al. 2012]. Activation of these gene targets needs PIF function, as the quadruple pifq mutant suppresses the BRZ-insensitive phenotype of bzr1-1D mutants and leads to short hypocotyls, as in pifq plants. Constitutive bzr1-1D mutants are insensitive to BRZ in darkness but show a hypersensitive response to this inhibitor in the light, in contrast to bes1-D mutants, reported to display also a BRZ-insensitive response in the light. Here we show that light-grown bes1-D seedlings still respond to BRZ by an inhibition of hypocotyl elongation and reduced PIL1 and PRE5 gene expression, suggesting that BIN2 phosphorylation and destabilization of PIF4 mediates this additional response to BRZ. In line with this model, expression of the PIF41A mutant protein in bes1-D plants resulted in tall plants that are fully insensitive to BRZ and show elevated levels of expression of the PIL1, XTR7, and PRE5 genes in the presence of this inhibitor. These results demonstrate that BR signaling not only activates BES1 and BZR1 but also promotes stabilization of the PIF4 factor, a concerted action of these two families of regulators being required for elongation growth.

PIF4 and BES1/BZR1 accumulation is required for cell elongation

Consistent with a role of BIN2 in regulating PIF4 stability, the bzr1-1D mutation was shown to suppress the
dwarf phenotype of bri1-116 seedlings in the dark but not in the light. Likewise, overexpression of the wild-type PIF4 protein increases the hypocotyl length of bzr1-1D bri1-116 plants [Oh et al. 2012], but these seedlings are still much shorter than the PIF4OX lines. Enhanced PP2A activity in PP2A B subunit overexpressers, on the other hand, leads to a reduced sensitivity to BRZ similar to that of the bzr1-1D mutation but is unable to suppress the weak bri1-5 allele even though these plants accumulate the dephosphorylated BZR1 protein [Tang et al. 2011].

The BZR1–BIN2 interaction is mediated by a small docking motif (DM) located near the C-terminal end of the BZR1 protein [Peng et al. 2010]. DM deletion completely eliminates BZR1 phosphorylation and leads to increased levels of accumulation of the BZR1(ΔDM)-GFP protein. However, as we observed for the PIF4A mutation, BZR1 (ΔDM) suppresses only the weaker phenotype of bin2-1 heterozygote mutants [Peng et al. 2010]. Thus, these findings support a role of BIN2 in PIF4 and BES1/BZR1 phosphorylation, nuclear accumulation of the nonphosphorylated forms of both proteins being required for growth.

The concerted action of PIFs and BES1/BZR1 modulates rhythmic hypocotyl growth

Although it is widely accepted that PIFs accumulate only in darkness, we show that the PIF4 protein follows a pattern of accumulation similar to that of its transcript. We generated pif4pif5 lines expressing the PIF4-HA or PIF4A-HA proteins under the control of its native promoter and selected lines with wild-type levels of these transgenes. pIF4::PIF4-HA lines had a hypocotyl length and expressed the PIL1 and PRE5 genes to levels similar to those of the Col-0 plants [Fig. 7; Supplemental Figs. S7, S12]. pIF4::PIF4A-HA lines, in contrast, were much taller and showed increased levels of expression of these genes (Fig. 7). pIF4::PIF4A plants are actually taller than the bes1-D mutant and, in the presence of BRZ,

Figure 6. Mutation of the conserved BIN2 phosphorylation motif increases the half-life of the PIF4A protein and leads to continuous growth. [A] Analysis of the half-life of the PIF4 and PIF4A proteins. Transgenic pIF4::PIF4-HA pif4pif5 and pIF4::PIF4A-HA pif4pif5 seedlings were grown in SD for 5 d and treated for 12 h with 0.5 μM BRZ before starting the assay. Three hours before lights on, seedlings were changed to 0.5 μM BRZ (mock) or 1 μM BL (BL) medium, and 50 μM CHX was added 1 h later. Seedlings were kept in darkness and harvested at different times after CHX application, as indicated. Western blot analyses of these samples showed that the wild-type PIF4 protein is destabilized after 30 min of CHX treatment, in contrast to PIF4A, which is still detected by 90 min of CHX application. BL treatment increased PIF4 stability but did not have any effect on the PIF1A protein.

Note that in BL-treated seedlings, the half-life of PIF4 becomes similar to that of PIF4A. [B] Stability of the PIF4 and PIF4A protein in the bin2-1 mutant background. pIF4::PIF4-HA pif4pif5 and pIF4::PIF4A-HA pif4pif5 lines were crossed with the bin2-1 mutant, and the accumulation levels and half-lives of these proteins were analyzed in 5-d-old plants as before. Before lights on, seedlings were transferred to MS medium with (+) or without (−) 50 μM CHX and incubated in darkness for 30 min. Western blot analysis showed that the wild-type PIF4 protein is destabilized in the bin2-1 mutant background but that constitutive activation of the BIN2 kinase does not affect PIF4A levels. [C] Diurnal growth patterns of Col-0, pIF4::PIF4-HA pif4pif5, pIF4::PIF4A-HA pif4pif5, and PIF4OX plants. Seedlings were grown in SD, and images were captured 3 d after germination at 1-h intervals. Five seedlings were measured for each genotype, and the growth rate was plotted as a function of time. Growth of pIF4::PIF4-HA pif4pif5 lines was similar to the wild type, with maximal hypocotyl elongation observed at dawn (Col-0). 35S::PIF4-HA lines [PIF4OX] elongated during the whole-night period, as previously described [Nozue et al. 2007]. pIF4::PIF4A-HA pif4pif5 lines displayed continuous growth, with the hypocotyls of these plants elongating also during daytime. Measurements were done twice with similar results. Light and dark hours are indicated by white and gray bars, respectively.
show increased levels of expression of the PIL1 and PRE5 transcripts, suggesting that PIF4 destabilization plays an important role in BRZ repression of hypocotyl growth. Time-course studies to analyze diurnal levels of accumulation of the PIF4 and PIF4A proteins showed that the wild-type PIF4 protein starts to accumulate at the end of the night, peaks during the day, and is reduced after transition to dark. PIF4A protein levels were higher than those of the wild-type protein, particularly during late night (ZT22) and immediately after lights on (ZT0–ZT2) (Fig. 5B). Strikingly, PIF4A plants displayed arrhythmic growth and, in contrast to PIF4 seedlings, elongated during daytime (Fig. 6C). This response is conferred by a mutation that makes PIF4 insensitive to BIN2-mediated destabilization, suggesting that diurnal oscillations in BR levels or signaling play a relevant role in setting the diurnal phase of hypocotyl growth. BR synthesis and response genes were shown to oscillate in short days, with a peak of expression that coincides with dawn (Michael et al. 2008; Yamashino et al. 2013). This time-of-day-specific expression plays a relevant role in setting the phase of maximal hypocotyl growth, as complementation of the bri1-116 mutant with the AtML1::BRI1 construct led to taller hypocotyls than the wild-type due to misexpression of the BRI1 gene during early night (Michael et al. 2008). This indicates that BR signaling plays an important role in hypocotyl elongation by providing robustness to the rhythmic growth pattern. Our data demonstrate that BIN2 redundantly regulates nuclear accumulation of BES1/BZR1 and the stability of PIF4, coactivator function of these factors thereby serving as a highly sensitive and robust mechanism for external cue integration, enabling the plant to adapt its growth and development to environmental changes.

Materials and methods

Plant materials and growth conditions

All of the plants used in this study were in the Col-0 ecotype. To generate the pPIF4::PIF4-HA pif4/pif5 transgenic lines, a geno-
mic fragment, including a 2.4-kb promoter region and the full-length PIF4 coding sequence without stop codon, was amplified with the pENTRY-promoter-I and PIF4_YFPr primers and cloned into the pENTRY S/D-TOPO [Invitrogen] vector. The pPIF4:PIF41A construct was obtained by substituting the NcoI–AgeI fragment of the pPIF4:PIF4 TOPO plasmid with the corresponding fragment, including the PIF41A mutation. These genomic regions were mobilized by LR clonase into the pGBW13-pZFP destination vector and transformed via Agrobacterium into the pif4pif5 mutant. bes1-D PIF41A plants were generated by crossing the pPIF4:PIF41A-HA plants into the bes1-D pif4pif5 mutants.

Seeds were transferred to vertical MS plates supplemented with the different chemical treatments and grown in darkness or continuous red light for 5–7 d, as specified. Plates were photographed, and the ImageJ software was used to measure the seedlings' hypocotyl length. Diurnal growth and protein accumulation studies were performed in plants grown in short days (8 h light/16 h dark).

**Protein interaction assays**

The full-length BIN2, PIF4, and BES1 coding regions were cloned into the pENTRY S/D-TOPO vector for recombination into the yeast and plant expression vectors. Yeast two-hybrid assays were performed with the GATEWAY-modified pGBK7 and pGADT7 vectors [Clontech]. Both bait and prey constructs were transformed into AH109 cells and selected on SD-LTHA medium. For pull-down assays, 35S-Met-radiolabeled PIF4 and BES proteins were synthesized with the TNT T7-coupled reticulocyte lysate system [Promega] and incubated with glutathione-Sepharose beads [GE Healthcare] with the bound GST and GST-BIN2 proteins. For co-IP studies, extracts of N. benthamiana leaves expressing the BIN2-HA and PIF4-GFP proteins were incubated with anti-HA magnetic beads (μMACS epitope tag, Miltenyi Biotec), and 15 μL [anti-HA] and 35 μL [anti-GFP] of the immunoprecipitated fraction were used for detection of the proteins. The BIN2 and PIF4 coding regions were fused to the N-terminal and C-terminal YFP fragments of the YFN43 and YFC43 vectors and co-infiltrated into N. benthamiana leaves. Leaves were observed 2 d after infiltration.

**In vitro kinase and phosphorylation assays**

PIF4-His, PIF41A-His, MBP-BES1, and BIN2-GST fusion proteins were purified using glutathione agarose [Clontech], amylose agarose [New England Biolabs], or Ni-NTA agarose [Qiagen] beads. For in vitro kinase assays, PIF4-His, MBP-BES1, and GST-BIN2 proteins were co-incubated with 32P-γ-ATP for 40 min at 37°C. Two micromolar and 20 μM of the BIN2 kinase inhibitor bikinin [Calbiochem] were added to the incubation mix for specific inhibition. Extracts of mock-, BL-, and BRZ-treated seedlings were incubated for 1 h at 37°C with or without CIP [New England Biolabs] to assay for in vivo protein phosphorylation.

**qRT-PCR gene expression analysis**

Total RNA was extracted by using the high pure RNA isolation kit [Roche]. The SuperScript II reverse transcriptase [Invitrogen] was used for cDNA synthesis, and quantitative real-time RT–PCR amplification was performed in the 7500 real-time PCR system [Applied Biosystems], following the manufacturer’s recommendations. PPA2 was used as an internal control. Gene-specific primers are listed in Supplemental Table 1.

**Confocal microscopy**

GFP fluorescence of the split YFP in BiFC-infiltrated N. benthamiana leaves or in roots of the transgenic 35S:PIF4-GFP and 35S:PIF41A-GFP lines was imaged using an inverted Leica TCS SP5 spectral confocal microscope. Fluorescence was excited with a 488-nm argon laser, and emission images were collected in the 500- to 600-nm range.

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**GENES & DEVELOPMENT**

**1694**
BR-dependent phosphorylation modulates PIF4 transcriptional activity and shapes diurnal hypocotyl growth

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