Interaction between BZR1 and PIF4 integrates brassinosteroid and environmental responses

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Plant growth is coordinately regulated by environmental and hormonal signals. brassinosteroid (BR) plays essential roles in growth regulation by light and temperature, but the interactions between BR and these environmental signals remain poorly understood at the molecular level. Here, we show that direct interaction between the dark- and heat-activated transcription factor phytochrome-interacting factor 4 (PIF4) and the BR-activated transcription factor BZR1 integrates the hormonal and environmental signals. BZR1 and PIF4 interact with each other in vitro and in vivo, bind to nearly 2,000 common target genes, and synergistically regulate many of these target genes, including the PRE family helix-loop-helix factors required for promoting cell elongation. Genetic analysis indicates that BZR1 and PIFs are interdependent in promoting cell elongation in response to BR, darkness or heat. These results show that the BZR1–PIF4 interaction controls a core transcription network, enabling plant growth co-regulation by the steroid and environmental signals.

In plants, cell elongation and seedling morphogenesis are controlled by multiple environmental factors and endogenous hormones, including light, temperature, brassinosteroid (BR), gibberellin (GA) and auxin. How these signals regulate largely overlapping cellular and physiological responses through distinct signalling pathways remains an outstanding question in plant biology. In particular, BR is required for hypocotyl elongation responses to dark, shade and high temperature in Arabidopsis. How BR mediates or modulates the environmental responses is poorly understood at the molecular level.

Light switches the developmental programme of seedlings from skotomorphogenesis to photomorphogenesis, causing inhibition of hypocotyl elongation, cotyledon opening and expansion, chloroplast development and a switch from heterotrophic to photoautotrophic growth. Light acts through a suite of photoreceptors, among which the red light-activated photoreceptor phytochromes directly interact with the basic helix-loop-helix (bHLH) transcription factors named phytochrome interacting factors (PIFs). PIFs accumulate in the dark to promote skotomorphogenesis, but are phosphorylated and degraded on light activation of phytochromes. The activities of PIFs are also regulated by GA, the circadian clock and temperature. Thus, PIFs are considered key transcription factors that integrate multiple hormonal and environmental signals.

BR is known to play a key role in light and temperature regulation of plant development, as the BR mutants, such as de-etiolated 2 (det2), show light-grown morphology and express light-induced genes in the dark, and are defective in hypocotyl elongation responses to high temperature and shade. BR signalling is mediated by the BRI1 receptor kinase and its downstream signal transduction cascade that leads to dephosphorylation and activation of the BZR1 family transcription factors. Constitutive active forms of BZR1, due to either increased dephosphorylation by protein phosphatase 2A (PP2A) or reduced binding by the 14-3-3 proteins (BZR1-1D) or reduced binding by the 14-3-3 proteins (BZR1-S173A), suppress the photomorphogenesis-in-the-dark phenotypes and most of the gene expression changes of the BR-deficient or BR-insensitive mutants, indicating that BR promotes skotomorphogenesis through BZR1.

The crosstalk between the light and BR pathways is believed to occur at or downstream of BZR1, because light does not significantly affect the levels of BR and BZR1. Indeed, BZR1 modulates the expression levels of many light-signalling components. In addition, genome-wide protein–DNA interaction analysis revealed BZR1 binding to the promoters of a significant portion of light-regulated genes, suggesting that BR and light signals converge at the promoters of common target genes through direct interaction between BZR1 and some light-signalling transcription factors. Here, we demonstrate that PIF4 directly interacts with BZR1, and together they programme a central transcriptional network that controls cell elongation and seedling photomorphogenesis. Our genetic evidence

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indicates that this interaction is a key mechanism for BR-dependent growth responses to light and temperature.

RESULTS
BZR1 interacts with PIF4
To test if BZR1 interacts with any light-signalling transcription factors, we carried out transient bimolecular fluorescence complementation (BiFC) assays in tobacco (Nicotiana benthamiana), by co-expressing a BZR1 fused to the carboxy-terminal half of yellow fluorescent protein (BZR1–cYFP) and light-signalling transcription factors fused to the amino-terminal half of YFP (nYFP). As shown in Fig. 1a, a strong YFP fluorescence signal was observed in the nucleus when BZR1–cYFP was co-transformed with PIF4–nYFP and PIF1–nYFP, indicating specific BZR1 interactions with PIF4 and PIF1. Co-immunoprecipitation assays using transgenic plants expressing BZR1–cYFP (cyan fluorescent protein) and PIF4–Myc from their native promoters confirmed their interaction in vivo (Fig. 1b). In vitro pulldown assays showed that maltose-binding protein (MBP)–BZR1 interacted with glutathione S-transferase (GST)–PIF4 and GST–PIF1 but not GST alone (Fig. 1c), and GST–PIF4 interacted with full-length MBP–BZR1, MBP–BZR2 and the N-terminal DNA-binding domain but not the C-terminal fragment of BZR1 (Fig. 1d,e,g), whereas MBP–BZR1N interacted with both the N-terminal fragment and bHLH domain but not the C-terminal fragment of PIF4 (Fig. 1f,h). The results indicate that BZR1 and PIF4 interact through their DNA-binding domains and the N-terminal domain of PIF4.

BZR1 and PIFs act interdependently in promoting hypocotyl elongation
We examined whether BR response requires PIFs. Members of the PIF family play overlapping roles in promoting skotomorphogenesis and cell elongation. The quadruple mutant (pifq) lacking four PIFs (PIF1, also known as PIL5, PIF3, PIF4 and PIF5, also known as PIL6) exhibits a constitutive de-etiolation phenotype in the dark, characterized by short hypocotyl and open cotyledons, similar to the BR-deficient or BR-insensitive mutants. The pifq mutant was less sensitive to exogenous brassinolide (BL) (Fig. 2a), suggesting that the loss of PIFs compromises BR response. However, the pifq single mutant responded to BRZ similarly to wild type (Fig. 2b), indicating redundant functions of PIFs with regard to BR response. The bZR1-1D gain-of-function mutation causes constitutive dephosphorylation of BZR1 by PP2A (ref. 22) and a BRZ-resistant phenotype. However, the pifq:bZR1-1D quintuple mutant had similar short hypocotyls as pifq grown on the medium with or without BRZ (Fig. 2c,d), suggesting that PIFs are required for the BZR1 promotion of hypocotyl elongation in the dark.

In contrast to the etiolation-promoting effect observed in the dark-grown bZR1-1D seedlings, the light-grown bZR1-1D mutant plants are weak dwarfs, with shorter hypocotyls and petioles than wild type (Fig. 2e,f). It seems that a light-inactivated factor, possibly PIFs, is required for BZR1 promotion of cell elongation. Indeed bZR1-1D increased hypocotyl elongation in the PIF4-overexpression (PIF4-OX) background under light (Fig. 2c), suggesting that BZR1’s function of promoting cell elongation requires PIF4.

Figure 1 BZR1 interacts with PIF4. (a) BiFC assay shows PIFs’ interaction with BZR1 in tobacco leaf cells. The images show overlays of fluorescence and light views. (b) Co-immunoprecipitation assay of BZR1 with PIF4. Plants expressing BZR1–cYFP from native BZR1 promoter and PIF4–Myc from native PIF4 promoter or plants expressing only PIF4–Myc were incubated at 28 °C for 8 h to accumulate PIF4 protein and treated with 100 nM BL for 1.5 h. BZR1–cYFP was immunoprecipitated using anti-GFP (green fluorescent protein) antibody and immunoblotted using anti-Myc or anti-GFP antibody. (c) PIFs directly interact with BZR1 in vitro. GST–PIF1 and GST–PIF4 were pulled down by MBP–BZR1 immobilized on maltose agarose beads and eluted and analysed by immunoblotting using anti-GFP antibody. (d) PIFs interact with both BZR1 and BZR2 in vitro. MBP–BZR1 and MBP–BZR2 were pulled down by GST–PIF4 immobilized on glutathione–agarose beads and then eluted and analysed by immunoblotting using anti-MBP antibody. (e) The N-terminal DNA-binding domain of BZR1 interacts with PIF4 in vitro. Various fragments of BZR1 fused to MBP were pulled down by GST–PIF4 and then eluted and analysed by immunoblotting using anti-MBP antibody. (f) Both N-terminal and bHLH domains of PIF4 interact with BZR1 in vitro. Various fragments of PIF4 fused to GST were pulled down by MBP–BZR1N and then eluted and analysed by immunoblotting using anti-GST antibody. (g,h) Box diagrams of the various fragments of BZR1 (g) and PIF4 (h) used in the pulldown assays in e and f. The black boxes indicate the DNA-binding domains. The numbers indicate the amino acids. Uncropped images of blots/gels are shown in Supplementary Fig. S7.

To test whether PIF4 promotion of cell elongation also requires BR signalling and active BZR1, we introduced PIF4-OX into the bRIL-116 single mutant, in which BZR1 is phosphorylated and inactive,
and the bri1-116;bzr1-1D double mutant, in which BZR1 is active. Whereas bzr1-1D suppressed the bri1-116 de-etiolation phenotype in the dark (Fig. 2f), the light-grown bri1-116;bzr1-1D showed similar short hypocotyls as bri1-116 (Fig. 2f;g; ref. 23), consistent with bzr1-1D being unable to promote cell elongation under light. Overexpression of PIF4 increased the hypocotyl length of bzr1-1D;bri1-116 but not of bri1-116 under light (Fig. 2f;g), demonstrating that both PIF4 and BZR1 are required for hypocotyl elongation. The pifq mutant showed normal BZR1 accumulation and phosphorylation status both before and after BR treatment (Supplementary Fig. S1), indicating that PIF4 does not affect BR signalling upstream of BZR1. Together, these results demonstrate that the BZR1–PIF complex is required for cell elongation and skotomorphogenesis; both degradation of PIFs by light signalling and inactivation of BZR1 by reduced BR signalling decrease the BZR1–PIF dimer formation and promote photomorphogenesis.

BZR1 and PIF4 bind to overlapping genomic targets

To understand the functions of BZR1–PIF4 interaction in regulating genome expression, chromatin immunoprecipitation sequencing (ChIP-seq) was carried out using transgenic plants expressing PIF4–myc from the PIF4 promoter (pPIF4::PIF4–myc) in the pifq mutant background, with the non-transgenic wild-type plants as a negative control. PIF4 binding to known targets (HFR1 and PUL; ref. 14) was detected in the PIF4–myc ChIP DNA sample but not in the control (Supplementary Fig. S2a). Analyses of the ChIP-seq data with the statistical software CisGenome28 and PRI-CAT (ref. 29) identified 3,510 and 4,573 PIF4-binding peaks, respectively (Fig. 3a). Among them, 3,186 peaks were identified by both statistical methods and thus considered as the high-confidence PIF4-binding peaks and used for further analysis. About 55% of PIF4-binding peaks were associated with at least one PIF-regulated gene whose expression is affected in the pifq;pif5 or pifq mutants according to previously published microarrays11,12,30–32 or our RNA-seq data (Fig. 3a). The 3,186 high-confidence PIF4-binding peaks were linked to 4,363 neighbour genes that were considered as high-confidence PIF4-target genes (Supplementary Table S1), of which 1,537 were PIF-regulated genes (58% genes downregulated and 35% genes upregulated by PIFs) and thus considered as PIF-regulated PIF4-target genes (PRPT; Fig. 3b and Supplementary Fig. S2b and Tables S2 and S3).

Most of the PIF4-binding peaks were in the promoter regions (within −5 kb from the transcription start site; Fig. 3c), consistent with the PIF4 function as a transcription factor. The distributions of PIF4-binding sites were similar between PIF-activated or PIF-repressed genes, suggesting that the up- or downregulation is independent of the binding location (Fig. 3c). Consistent with previous studies showing PIFs binding to the G-box element13,34, the G-box motif (CACGTG) is the most enriched cis element in the PIF4-binding sites, and the E-box motif (CACATG) was

![Figure 2](image-url)
also enriched but to a lesser degree, whereas the frequency of the CTATAG motif as the negative control was not increased near the PIF4-binding peaks (Fig. 3d and Supplementary Fig. S2c). Similar cis-element enrichment was found in PIF-activated or repressed genes (Supplementary Fig. S2c).

The target genes of PIF4 include over half (51.7%) of the BZR1-target genes identified by ChIP microarray (Fig. 3e). These target genes of both BZR1 and PIF4 contain a higher portion of light-regulated genes than the target genes of either BZR1 or PIF4 alone (Fig. 3e). When plotted along the gene structure, most of the binding peaks of BZR1 and PIF4 were clustered along the diagonal line (Fig. 3f), suggesting that they bind to nearby or the same cis elements (Fig. 3g).

Motif analysis showed that the G-box (CACGTG), which contains two inverted repeats of the core of the BZR1-binding site (CGTG; ref. 27), was the most enriched motif in the regions bound by both BZR1 and PIF4 (Supplementary Fig. S2d,e). To test if BZR1 and PIF4 bind to the same promoter elements at the same time or exclusively of each other, chromatin from transgenic Arabidopsis expressing both BZR1–Myc and PIF4–YFP was immunoprecipitated sequentially using anti-Myc and anti-YFP antibodies, and then analysed by quantitative PCR (qPCR). The results show a high enrichment of the BZR1 and PIF4 common target promoters, indicating that BZR1 and PIF4 co-occupy these promoters in vivo (Fig. 3h). These results, together with in vitro data showing direct interaction between their DNA-binding domains, suggest that BZR1 and PIF4 form a heterodimer to bind to promoters of the common target genes (Fig. 1).
We carried out RNA-seq analysis to determine whether genes are controlled independently or interdependently by PIFs and BZR1. Wild-type, bzrl-1D, pifq and pifq;bzrl-1D plants were grown on the medium containing 2 μM BRZ (to inactivate wild-type BZR1) in the dark for five days. RNA-seq analyses identified 2,151 genes affected more than twofold by bzrl-1D mutation when compared with wild type (BZR1-regulated genes) (Fig. 4d and Supplementary Table S4), and 3,176 genes affected by the pifq mutation in the bzrl-1D background (pifq;bzrl-1D versus bzrl-1D, PIF-regulated genes; Fig. 4d and Supplementary Table S5). About 59% (1,279) of the BZR1-regulated genes are also regulated by PIFs, mostly in the same direction (correlation coefficient = 0.87; Fig. 4e). There are more genes activated than repressed by BZR1 and PIFs, and this is particularly true for the co-target genes of BZR1 and PIF4 (Supplementary Fig. S4). The overall effects of bzrl-1D on the gene expression are significantly diminished in the pifq background when compared with the wild-type background (Fig. 4f), with differential expression of about 78% of the 2,151 bzrl-1D-affected genes abolished or reversed in the pifq background (Fig. 4d,f). Among the 3,176 genes affected by pifq in the bzrl-1D background, 2,039 genes (64%) were unaffected or affected in opposite ways by pifq in the wild-type background, which lacks BZR1 activity when grown on the BRZ medium (Supplementary Fig. S5). These results demonstrate that BZR1 and PIFs regulate a large portion of downstream genes interdependently on each other but also regulate some genes independently.

We carried out quantitative PCR with reverse transcription (qRT–PCR) of several BZR1–PIF4 common target genes in the bzrl-1D, pifq and bzrl-1D;pifq mutants to confirm their independent or interdependent regulation by BZR1 and PIF4. As shown in Fig. 4g, the
expression levels of the BZR1-activated SAUR15, IAA19, PRE and ACS5 genes and BZR1-repressed GER1 and FAD5 genes were less affected by bzip1-1D in the pifq background than in the wild-type background, suggesting that the optimal BZR1 transcriptional activity on these target genes requires PIFs. In contrast to pifq, PIF4 overexpression had similar effects on these genes as bzip1-1D, and together PIF4-OX and bzip1-1D showed synergistic effects (Fig. 4h). On the other hand, inhibiting BR synthesis using BRZ diminished the effects of PIF4-OX on these genes, whereas bzip1-1D partly overcame the inhibitory effects of BRZ (Fig. 4i), indicating that BZR1 is required for the PIF4 regulation of these genes. These results confirm that BZR1 and PIF4 cooperatively regulate these common target genes. The qRT–PCR analysis also confirmed the PIF-independent actions of BZR1 on the BR biosynthesis genes CDP, BR6O2X and DWF4 (Fig. 4g,h).

PREs promote cell elongation downstream of BZR1 and PIF4

Among the BZR1–PRE5–coregulated genes (Fig. 4g), the PRE family of small HLH proteins has been characterized as positive regulators of cell elongation. Among the six PRE members, PRE1, PRE5 and PRE6 were identified as PIF4 targets in the ChIP-seq analysis (Supplementary Fig. S6a), and confirmed by ChIP–qPCR to be direct targets of both BZR1 and PIF4 (Supplementary Fig. S6b,c). The expression levels of PRE genes were markedly reduced in pifq but only slightly reduced in the pifq single mutant, suggesting that PIF4 and further PIFs redundantly activate these PRE genes (Supplementary Fig. S6d). Expression of these PRE genes was activated by bzip1-1D in a PIF-dependent manner (Fig. 4g), synergistically activated by bzip1-1D and PIF4-OX (Fig. 4h), and activated by PIF4-OX in a BR/BZR1-dependent manner (Fig. 4i). Transient assays of a pPRE5::Luc reporter gene further confirmed that BZR1 and PIF4 synergistically activate PRE5 (Fig. 5a).

We generated a transgenic line in which four PRE genes (PRE1, 2, 5, 6) were knocked down using artificial microRNA (pre-amiR; Fig. 5b). The pre-amiR plants showed dwarf phenotypes similar to BR-deficient or BR-insensitive mutants (Fig. 5c), and a reduced response to BR treatment (Fig. 5d,e). Suppression of PRE genes also increased the sensitivity to BRZ in both wild-type and bzip1-1D background (Fig. 5f). Knockdown of PRE genes also enhanced the plant sensitivity to light (Fig. 5g), whereas overexpression of PRE1 suppressed the dwarf phenotype of pifq (Fig. 5h). These results demonstrate that PREs are key components promoting cell elongation downstream of BZR1 and PIF4.

High temperature promotion of hypocotyl elongation requires both BZR1 and PIF4

BR is required for Arabidopsis hypocotyl elongation in response to high temperature, which is mediated by an increase of PIF4 expression. We thus tested whether BZR1 acts together with PIF4 to promote hypocotyl elongation under high temperature. The high-temperature-induced hypocotyl elongation was abolished by the BR biosynthesis inhibitor propiconazole (PPZ) in wild type but not in the bzip1-1D mutant (Fig. 6a,b), indicating that BR activation of BZR1 is required for the high-temperature-induced hypocotyl elongation. The pifq;bzip1-1D mutant showed no response to high temperature, consistent with PIF4’s essential role in hypocotyl response to high temperature (Fig. 6a,b). As reported previously, high temperature increased the PIF4 protein accumulation (Fig. 6c). In contrast, BZR1 was not obviously affected by temperature (Fig. 6d), indicating that high temperature mainly increases the level of PIF4, which promotes hypocotyl elongation in a BZR1-dependent manner. Consistent with a BZR1-dependent regulation, the expressions of several BZR1 and PIF4 co-target genes including PREs were increased by high temperature in wild type and bzip1-1D; bzip1-1D but not in bzip1-1D (Fig. 6e). The hypocotyl...
The interactions between BZR1 and PIF4 seem to be promoter specific, and BZR1 and PIF4 each also controls large numbers of unique targets. This potentially enables differential regulation of various processes by BR and environmental signals. The genome-wide analysis confirmed that the major functions of BZR1–PIF4 are activating genes involved in cell elongation while repressing the transcription pathways for chloroplast development. In addition to cell-wall-related genes, BZR1–PIF4 promotion of cell elongation seems to also require the downstream PRE family of HLH factors, which are homologous to the human Inhibitor of DNA-binding (Id) protein. Previous studies have shown the major function of the PREs in promoting growth by antagonizing other bHLH factors including IBH1, AIF1 and PAR1 (refs 35,36,38). We show here that PREs are co-activated by BZR1 and PIF4, and they are required for the BZR1/PIF4-mediated hypocotyl elongation responses to BR, darkness and high temperature. A recent study showed that PAR1 interacts with PIF4 (ref. 39); therefore, the transcription activation of PREs may also activate a feedback loop, further activating PIF4. BZR1 and PIFs seem to act at the centre of a complex transcriptional network that controls cellular growth and development of photosynthetic apparatus.

Interestingly, BZR1 and PIF4 tend to regulate photosynthetic/chloroplast genes indirectly through their target transcription factors such as GLK1 and GLK2, which are key transcription factors regulating expression of photosynthetic apparatus. GLK1 is a

Figure 6 High temperature promotion of hypocotyl elongation requires both BZR1 and PIF4. (a,b) Both BZR1 and PIF4 are required for high-temperature promotion of hypocotyl elongation. Seedlings were grown either on mock (−PPZ) or 2 μM PPZ (+PPZ) at 20 °C or 28 °C for seven days. Numbers indicate the ratios of hypocotyl lengths of seedlings at 28–20 °C. Error bars indicate the s.d. (n = 10 plants). (c) PIF4 protein accumulates at high temperature. Seedlings expressing PIF4–Myc from native PIF4 promoter were grown at 20 °C for five days and transferred to 28 °C for 1, 4 and 24 h. (d) BZR1 accumulation and phosphorylation status are not significantly affected at high temperature. Seedlings expressing BZR1–CFP from native BZR1 promoter were grown at 20 °C for five days and transferred to 28 °C for 1, 4 and 24 h. (e) Both BZR1 and PIF4 are required for high-temperature-induced gene expressions. Seedlings were grown at 20 °C for four days and transferred to 28 °C for 24 h (28) or kept at 20 °C (20). Relative gene expression levels were normalized to that of PP2A. Similar results were obtained in two independent experiments. (f) The pre-amiR plants are defective in the high-temperature promotion of hypocotyl elongation. Seedlings were grown at 20 °C or 28 °C for seven days. Error bars indicate the s.d. (n = 10 plants). Uncropped images of blots/gels are shown in Supplementary Fig. S7.

DISCUSSION

Signalling crosstalk is considered important for cellular decision-making, but few examples of direct crosstalk between signalling pathways have been elucidated in plants, although plants are expected to have more complex signalling system than animals. Genetic and physiological studies have suggested a close relationship between BR- and light-signalling pathways, yet evidence for direct crosstalk has been elusive. Here we show that light-regulated PIF4 interacts directly with BR-regulated BZR1. Together they bind to thousands of common target genes in the genome, and interdependently control gene expression and seedling morphogenesis. The functional interdependence between PIF4 and BZR1 explains why BR is required for cell elongation responses to multiple environmental signals that activate PIFs, including darkness, shade and high temperature. This relation is also consistent with the light-dependent phenotype of bzr1-1D (ref. 27). The interaction seems conserved among further members of the BZR1 and PIF families. Although bes1-D was reported to show a light-independent long-hypocotyl phenotype, the difference could be due to different genetic background or a possibly light-independent activity of BEs1. Our strong genetic and molecular evidence indicates that BZR1–PIF4 interaction is a key mechanism for coordination of growth regulation by BR, light and temperature.

Most of the BZR1–PIF4-co-regulated target genes were positively regulated by both BZR1 and PIF4, suggesting that the BZR1–PIF4 heterodimer functions as a transcription activator. However, the interactions between BZR1 and PIF4 seem to be promoter specific, and BZR1 and PIF4 each also controls large numbers of unique targets. This potentially enables differential regulation of various processes by BR and environmental signals. The genome-wide analysis confirmed that the major functions of BZR1–PIF4 are activating genes involved in cell elongation while repressing the transcription pathways for chloroplast development. In addition to cell-wall-related genes, BZR1–PIF4 promotion of cell elongation seems to also require the downstream PRE family of HLH factors, which are homologous to the human Inhibitor of DNA-binding (Id) protein. Previous studies have shown the major function of the PREs in promoting growth by antagonizing other bHLH factors including IBH1, AIF1 and PAR1 (refs 35,36,38). We show here that PREs are co-activated by BZR1 and PIF4, and they are required for the BZR1/PIF4-mediated hypocotyl elongation responses to BR, darkness and high temperature. A recent study showed that PAR1 interacts with PIF4 (ref. 39); therefore, the transcription activation of PREs may also activate a feedback loop, further activating PIF4. BZR1 and PIFs seem to act at the centre of a complex transcriptional network that controls cellular growth and development of photosynthetic apparatus.
target gene of both BZR1 and PIF4, whereas GLK2 is a PIF4 target; both are repressed by BZR1, BZR2 (also known as BES1) and PIFs (refs 12,19,43). Of the 120 GLK1/2-activated genes identified by microarray analysis, 55 genes (46%) were repressed by BZR1 and/or PIFs (Supplementary Table S6), consistent with the repression of GLK1/2 by BZR1 and PIFs. Previous studies have shown that BZR1 inhibits photomorphogenesis by transcriptional repression of light-signalling components20, including phytochrome B as well as GATA2 and BZSI transcription factors21,22,23. As such, BZR1 not only directly cooperates with the negative regulators (PIFs) but also transcriptionally represses the positive regulators of photomorphogenesis, providing two levels of control. These interactions at multiple levels support the importance of steroid regulation of light sensitivity. In addition to light and temperature, circadian rhythm and GA also control the activities of PIF factors13–15, and thus the responses to these signals may also depend on BR/BZR1. The BZR1–PIF4 module therefore seems to be the core of a complex network that integrates multiple endogenous and environmental signals.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

E.O. and J-Y.Z. carried out experiments. E.O. analysed data and wrote the manuscript. Z-Y.W. designed experiments, analysed data and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

Plant materials and growth conditions. A. thaliana plants were grown in a greenhouse with a 16-h light/8-h dark cycle at 22–24 °C for general growth and seed harvesting. All the plants were in Col-0 ecotype background. To generate PIIF4-overexpressing plants, the full length of the PIIF4 coding sequence was cloned into gateway compatible pEarleyGate 101 (ref. 41) and transformed into Col-0. To generate PRΔ knockdown mutants (pre-amir), we used WMD3 (Web MicroRNA Designer 3; ref. 45) to design an artificial microRNA sequence (5′-TTATGATTGCTATGCTCGT-3′) targeting PRE1, PRE2, PRE5 and PRE6 and transformed the artificial microRNA driven by the 35S promoter into Col-0. The preamp12 was provided by G. Choi (Korea Advanced Institute of Science and Technology).

Hypocotyl length measurements. Seeds sterilized by 70% (v/v) ethanol and 0.01% (v/v) Triton X-100 were grown on half-strength MS medium (PhytoTechnol-ogy Laboratories) supplemented with 0.8% phytoagar. After three days of incubation at 4 °C, seedlings were irradiated by white light for 6 h to promote germination and then incubated in specific light conditions for five days. For high-temperature experiments, seedlings were incubated at 20 °C for one day and incubated at 28 °C for five to six days. Seedlings were photocopied and hypocotyl lengths were measured by using ImageJ software (http://rsb.info.nih.gov/ij/).

Transient gene expression assays. Protoplast isolation and PEG transformation was carried out as described previously (tape method)46. Plasmid DNAs were extracted using the Qiagen Plasmid Maxi Kit according to the manufacturer’s instructions. 1 × 106 isolated mesophyll protoplasts were transformed with a total of 10 µg of DNA (35S:PIF4–GFP, 35S:BDZ1–GFP, 35S: Renilla luciferase and PRE5 promoter:firefly luciferase) and incubated overnight. Protoplasts were harvested by centrifugation and lysed in 50 µl of passive lysis buffer (Promega). Firefly and Renilla (as internal standard) luciferase activities were measured using a dual-luciferase reporter kit (Promega).

Co-immunoprecipitation assays. Plants expressing both BRZ1–CFP from native BRZ1 promoter (BRZ1p:BRZ1–CFP) and PIIF4–Myr from native PIIF4 promoter (PIIF4p:PIIF4–myr) or plants expressing only PIIF4–Myr were incubated at 28 °C for 8 h to accumulate PIIF4 protein and treated with 100 mM BtL 1.5 h. Harvested tissues were ground in liquid nitrogen, homogenized in immunoprecipitation buffer (50 mM Tris-HCl at pH 8.0, 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 3% glycero, 1 mM phenylmethylsulphonyl fluoride, 1× protease inhibitor) and sonicated four times to break the nuclei. After centrifugation at 20,000g for 10 min, 1 ml of the supernatant was incubated overnight with anti-GFP (5 µg) immobilized on protein A/G agarose beads. The beads were then washed three times with 1 ml of immunoprecipitation buffer and eluted samples were analysed by immunoblot.

Pulldown assays. GST–PIF4, GST–PIF1, GST–PIF4N1, GST–PIF2N, GST–PIF4C1, GST–PIF2C2, GST–PIF3C, GST, MBP–BRZ1, MBP–BRZ2, MBP–BZR1N, MBP–BRZ1C and MBP proteins were expressed in BL21 codon plus Escherichia coli cells and purified either using glutathione beads (GE Healthcare) or amyllose resin (NEB) according to the manufacturer’s protocol respectively. 1 µg of MBP–BRZ1-bound maltose agarose beads were incubated with either 1 µg of GST or GST–PIF4 or GST–PIF1 in 1× TEB buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) at 4 °C for 1 h. The beads were then washed three times with NETN buffer (200 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 20 mM Tris-HCl, pH 8.0), and eluted samples were analysed by immunoblotting using anti-GST antibody (GST (Z-5) antibody: sc-559; Santa Cruz) at 1:5,000 dilution. MBP–BRZ1, MBP–BRZ2, MBP–BZR1N, MBP–BZR1C and MBP were pulled down by GST–PIF4 immobilized on glutathione agarose beads and then eluted and analysed by immunoblotting using anti-MBP antibody (anti-MBP monoclonal antibody; NEB) at 1:5,000 dilution. GST–PIF4N1, GST–PIF2N, GST–PIF4C1, GST–PIF2C2, GST–PIF3C and GST were pulled down by MBP–BZR1N-terminal fragment immobilized on maltose agarose beads and then eluted and analysed by immunoblotting using anti-GST antibody.

Bimolecular fluorescence complementation (BiFC) assays. BiFC assays were carried out as described previously43. Briefly, agrobacterial suspensions containing PIF4–nYFP or PIF1–nYFP and BRZ1–cFP constructs were injected into the lower epidermis of tobacco leaves. The transplanted leaves were kept in the greenhouse for at least 36 h at 22 °C and fluorescence signals were visualized by using a spinning-disc confocal microscope (Leica Microsystems).

Gene expression analysis. Total RNA was extracted from five-day-old seedlings using the Spectrum Plant Total RNA kit (Sigma). M-MLV reverse transcriptase (Fermentas) was used to synthesize complementary DNA from the RNA. Quantitative real-time PCR (qPCR) was carried out using LightCycler 480 (Roche) and the Bioline SYBR Green Master Mix. PPA2 was used as an internal control. Gene specific primers are listed in Supplementary Table S7.

Chromatin immunoprecipitation (ChiP) assays. To generate PIIF4 native promoter-driven PIIF4–Myr (PIIF4p:PIIF4–myr) in the pifq mutant background, a PIIF4 genomic fragment including 2 kb upstream of the transcription start site was cloned into the gateway compatible pGWBI7 vector45 and transformed into the pifq mutant. A line showing the wild-type phenotype was selected and used for ChiP assay. The pIF4:PIIF4–myr/pifq plants were grown under white light for two weeks or in the dark for five days and cross-linked for 20 min in 1% formaldehyde under vacuum. The chromatin complex was isolated as previously described43 and sheared by sonication to reduce the average DNA fragment size to around 300 bp. The sonicated chromatin complex was immunoprecipitated by anti-Myc antibody (Myc-Tag (B2) mouse mAb; Cell Signaling). The beads were washed with low-salt buffer (50 mM Tris-HCl at pH 8.0, 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100), high-salt buffer (50 mM Tris-HCl at pH 8.0, 2 mM EDTA, 300 mM NaCl, 0.5% Triton X-100), LiCl buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA, 0.25 M LiCl, 0.5% NP-40, 0.5% deoxycholate) and TE buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA) and eluted with elution buffer (1% SDS, 0.1 M NaHCO3). After reverse cross-linking, the DNA was purified with a PCR purification kit (Fermentas) and analysed by ChiP-qPCR. Primers are listed in Supplementary Table S7.

ChiP-seq analysis. For ChiP-seq library construction, we used 10 ng of ChiP DNA pooled from three biological repeats to reduce sample variation. Blunting end, adapter ligation and amplification were carried out using Illumina Genomic DNA Sample Prep Kits according to the manufacturer’s protocol with small modifications. We used diluted (1:10) adapter and 150–400 bp gel-purified amplified DNA. Illumina Genome Analyser Ix was used for high-throughput sequencing of the ChiP-seq library. The raw sequence data were processed using Illumina sequence data analysis pipeline GAPipeline1.3.2. Sequences in Solexa FASTQ format were mapped to the Arabidopsis genome, TAIR9. Only unique mapping reads were used for calling PIIF4-binding peaks. Two-Sample-Peak-Calling in CisGenome with parameters Bin Size B = 50, Max Gap = 50, Min Peak Length = 100, Win Stat Cutoff C = 2.5, Local Rate Cutoff = 1e-005 and PRI-CAT with default parameters and cutoff (FDR < 0.05) were applied to define the PIIF4-binding peaks. Peaks detected in both statistical methods were considered as high-confidence binding peaks and used in further analysis. Two nearest neighbour genes flanking a binding site and genes that contain binding sites within the transcribed region were defined as PIIF4-target genes. The PIIF4-target genes were compared with previously published microarray data to identify PIIF-regulated PIIF4-target genes (PRPT; refs 11,12,3032).

For genomic distribution of PIIF4-binding peaks relative to gene structure, we divided the genome into three regions; 5 kb upstream of the transcription start site (TSS) to the TSS, the TSS to the 3′ end of the gene and the 3′ end of the gene to 1 kb downstream of gene and then calculated the frequency of binding peaks in these three regions. If a peak was located within 5 kb upstream of one gene and 1 kb downstream of another gene, the peak was counted in both regions. Peaks outside these regions were not included and peaks existing within 5 kb upstream of two different genes were counted twice.

To discover in vivo PIIF-binding motifs, DNA sequence of binding peaks were applied to MEME-ChIP with a maximum motif length of 10 (ref. 48). The motifs identified by MEME-ChIP were further analysed by comparing the frequencies of the motif in the binding peaks to those in Arabidopsis total genome (TAIR9, www.arabidopsis.org). The singular enrichment analysis method was used for GO analysis.

ChiP–reChiP assays. ChiP–reChiP assays were carried out as previously described40. Two-week-old 35S:BRZ1–myr:35S:PIF4–YFP double transgenic plants were crosslinked for 20 min in 1% formaldehyde under vacuum. Isolated chromatin complex was sheared by sonication to achieve an average DNA fragment size around 500 bp. The sonicated chromatin complex was immunoprecipitated by anti-Myc antibody (first ChiP), washed by the same buffer as for ChiP assays and eluted with 10 mM dithiothreitol. Eluted chromatin complex was diluted 20-fold with dilution buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1, 150 mM NaCl), immunoprecipitated again by anti-GFP antibody (second ChiP) and eluted with elution buffer (1% SDS, 0.1 M NaHCO3). After the second ChiP and reverse cross-linking, the DNA was purified with a PCR purification kit (Fermentas) and quantified by ChiP-qPCR.

RNA-seq analysis. Seedlings were grown on half-strength MS medium containing 2 µM BR2 in the dark for five days before harvesting. Total RNA was extracted from
five-day-old seedlings using the Spectrum Plant Total RNA kit (Sigma). Libraries were constructed using a TruSeq RNA sample preparation kit (Illumina) according to the manufacturer’s instruction. Total reads were mapped to the A. thaliana genome (TAIR9, www.arabidopsis.org) using the TopHat software. Read counts for every gene were generated using HTSeq with union mode. Differential expressed genes between samples were defined by DEseq (ref. 51), using fold change > 2 and \( P \)-value < 0.01.

**GEO accession numbers.** The ChIP-seq data used in this study may be viewed under GSE35315.

The RNA-seq data used in this study may be viewed under GSE37160.

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Figure S1 BZR1 phosphorylation status is not affected in *pifq* mutant. Seedlings were grown on the medium containing 2 μM BRZ for 4 days in the dark and then treated with either mock (M) or 100 nM BL for 2 hr. Endogenous BZR1 was detected by anti-BZR1 antibody. p-BZR1 : phosphorylated BZR1, BZR1 : de-phosphorylated BZR1.
Figure S2 PIF4 ChIP-seq analyses. (a) Representative PIF4 binding peaks. Peaks in the promoters of *HFR1* and *PIL1* were detected in the *PIF4-myc* sample, but not detected in the wild type control sample. (b) 1537 PIF-regulated-PIF4-target (PRPT) genes. Up indicates PIF-activated gene; Down indicates PIF-repressed gene; Complex indicates gene differently regulated by PIF in the different data set. (c) Number of enriched motifs per 1 kb in the PIF4 binding peaks. Enriched motifs frequency were not different between PIF-activated (up) and PIF-repressed (down) PIF4 target genes. Genomic indicates *Arabidopsis* total genome. (d) Sequence logo shows the most enriched motif in the BZR1 and PIF4 common binding regions. (e) Number of enriched motifs per 1 kb in the BZR1 and PIF4 common binding regions. Genomic indicates *Arabidopsis* total genome.
Figure S3 GO analyses of PIF-regulated PIF4 target genes (PRPT). Enriched GO (gene ontology) categories of PRPT compared with total *Arabidopsis* genes (Total). Blue numbers indicate *p*-value.
Figure S4 BZR1 and PIF4 co-targets are more likely to be activated by BZR1 and PIFs. Scatter plot of log2 fold change values in bzar1-1D/Col-0 or bzar1-1D/pifq;bzar1-1D RNA-Seq data for 103 BZR1 and PIF4 co-regulated co-targets genes. In the table, “All” indicates BZR1 and PIF4 co-regulated genes (1279) and “BZR1,PIF4 co-targets” indicates BZR1 and PIF4 co-regulated co-target genes (103). Numbers indicate number of genes.
Figure S5 PIF effect on gene regulation is dependent on BZR1 activity. (a) Comparison of BZR1-regulated genes (bzr1-1D vs WT(Col-0)) and PIF-regulated genes (bzr1-1D vs pifq;bzr1-1D and WT vs pifq) identified by RNA-Seq analysis. Differential expressed genes were defined by 2-fold difference between samples with p-value< 0.01. (b) Scatter plot of log2 fold change values of 3176 PIF-regulated genes in the bzr1-1D versus wild type background.
Figure S6 PREs mediate BR and light crosstalk downstream of BZR1 and PIF4. (a) PIF4 binding peaks in the promoters of PRE1, PRE5 and PRE6. (b) ChIP-qPCR using PIF4p::PIF4-myc shows PIF4 binding to PREs promoters. Enrichment of pulled DNA was calculated as ratio between PIF4-myc and wild type, normalized to that of PP2A coding region as an internal control. Similar results were obtained in two independent experiments. (c) BZR1 binds to PREs promoters. 5 days dark-grown BZR1p::bzr1-1D-CFP transgenic seedlings were used for ChIP assay. Enrichment of pulled DNA was calculated as ratio between bzr1-1D-CFP and wild type, normalized to that of PP2A coding region as an internal control. Error bars indicate s.d. of three independent experiments (n=3). (d) PREs are redundantly regulated by PIFs. Seedlings were grown in the dark for 4 days. Relative gene expression levels were normalized to that of PP2A. Error bars indicate s.d. of three independent experiments (n=3).
Figure S7 Full scan images of immunoblots.